REMARKS

Claims 25-31 are pending and there remain two outstanding issues. One is whether the Office may require restriction between nucleic acid sequences within a single claim. It is respectfully submitted that the Office may not properly restrict between the three nucleic acid sequences specified in each independent claim. The second is whether the claims have utility and whether the specification enables their use in accordance with 35 U.S.C. § 101 and 112, first paragraph. It is respectfully submitted that the claimed methods have well-established, specific, substantial and credible utilities and the specification teaches such uses.

Response to Restriction Requirements

In the Office action response filed January 31, 2003, the applicants elected the species of SEQ ID NO: 23 with traverse in response to the species election requirement set forth in paper no. 24 mailed December 31, 2002. In the Office action mailed May 6, 2003, the Office stated that the species election requirement was meant to be constructed as a restriction requirement and made the requirement final. The Office deemed for the first time in the Office action mailed May 6, 2003 that the election of SEQ ID NO: 23 was a requirement for restriction. Therefore, it is respectfully submitted that making the restriction requirement final was improper. Accordingly, it is respectfully requested that the Office withdraw the finality of the restriction requirement in the Office action mailed May 6, 2003 and consider the following remarks.

The claims are directed in part to processes for screening T-type calcium channel agonists and antagonists, where the channel is encoded by a nucleotide sequence which hybridizes to a nucleic acid comprising SEQ ID NO: 23, 25 or 27. It has long been held that the Office may not impose a restriction requirement on a single claim. *See In re Watkinson*, 14 USPQ.2d 1407 (Fed. Cir. 1990) citing *In re Weber*, 198 USPQ 328, 332 (CCPA 1978) and *In re Haas*, 198 USPQ 334, 336 (CCPA 1978). The courts have definitively ruled that the statute authorizing restriction practice (*i.e.* 35 U.S.C. § 121) provides no authority to impose a restriction requirement on a single claim, even if the claim presents multiple independently

patentable inventions. In these cases, the courts expressly ruled that there is no statutory basis for rejecting a claim for misjoinder, despite previous attempts by the Office to fashion such a rejection. As noted in *In re Weber*:

> The discretionary power to limit one applicant to one invention is no excuse at all for refusing to examine a broad generic claim, no matter how broad, which means no matter how many independently patentable inventions may fall within it.

In re Weber at 334.

Alleging that a particular claim represents multiple "patentably distinct" inventions is a *de facto* rejection of the patentability of the claim because the claim cannot issue as drafted. In this regard the courts noted:

> As a general proposition, an applicant has a right to have each claim examined on the merits. If an applicant submits a number of claims, it may well be that pursuant to a proper restriction requirement, those claims will be dispersed to a number of applications. Such action would not effect the rights of the applicant eventually to have each of the claims examined in the form he considers to best define his invention. If, however, a single claim is required to be divided up and presented in several applications, that claim will never be considered on the merits. The totality of the resulting fragmentary claims would not necessarily be the equivalent of the original claim. Further, since the subgenera would be defined by the examiner, rather than by the applicant, it is not inconceivable that a number of fragments would not be described in the specification.

See In re Weber , *supra,* emphasis added.

Instead of improperly imposing a restriction requirement on a given claim, the Office may limit initial examination to a "reasonable number" of species encompassed by the claim (*see* 37 C.F.R. § 1.146). This practice strikes an appropriate balance between administrative concerns of the Office and the clear constitutional and statutory rights of the inventor to claim an invention as it is contemplated. *See* MPEP at § 803.02; *In re Wolfrum*, 179 USPQ 620 (CCPA 1973); and *In re Kuehl*, 177 USPQ 250 (CCPA 1973). Unlike a restriction requirement, a species election does not preclude an applicant from pursuing the original form of a claim in subsequent

prosecution nor does it force an applicant to file multiple divisional applications that are incapable of capturing the intended scope of the application. Here, it should be clear that the added cost of filing and prosecuting multiple patent applications does not strike an appropriate balance between the administrative concerns of the Office and the applicants' statutory rights as inventors.

It also respectfully is submitted that there is no undue search burden on the Office when performing a database search for the pending claims. A search that covers one of the specified nucleic acid sequences should be broad enough to cover the two other related nucleic acid sequences. As one search should cover all three nucleotide sequences claimed, there should be no undue search burden on the Office. Accordingly, the applicants again provisionally elect claims directed to the use of the α_1 subunit species encoded by a nucleotide sequence that hybridizes to a nucleic acid comprising SEQ ID NO: 23, and request, respectfully, reconsideration of the restriction requirement.

The Claimed Subject Matter has a Well-Established Utility

The Office rejected the pending claims as the specification allegedly does not provide a specific, substantial or credible utility for the claimed subject matter. These rejections under 35 U.S.C. §§ 101 and 112, first paragraph respectfully are traversed. There are at least six features underscoring the utility of the claimed screening assays:

1. the α_1 subunits encoded by nucleotide sequences that hybridize to a nucleic acid comprising SEQ ID NO: 23, 25 or 27 are functional full-length subunits and the specification teaches a person of ordinary skill in the art how to use the specified α_1 subunits screening assays;

2. publications at the time the priority application was filed demonstrate T-type calcium channel assays had a well-established utility as they were useful for identifying compounds that treat diseases such as hypertension, stroke, epilepsy, heart disease and cancer;

3. a declaration submitted by Dr. Snutch, which must be considered by the Office, demonstrates the claimed screening methods are useful for identifying molecules that treat T-type calcium channel related diseases;

4. due to the facts delineated in items 1, 2 and 3, the screening assays have a well-established, specific, substantial and credible utility for identifying compounds useful for treating diseases enumerated in the specification;

5. little or no experimentation is required beyond the claimed processes to identify compounds that treat diseases listed in the specification; and

6. the Office already has allowed claims directed to T-type channels, proving that claims directed to their use have utility.

The following describes these features in greater detail.

First, the α_1 subunits encoded by nucleotide sequences that hybridize to a nucleic acid comprising SEQ ID NOs: 23, 25 or 27 form functional full-length calcium channels (*see e.g.*, specification on page 5, line 1, and page 24, lines 10-18). Although the properties of these channels may be modulated by co-expression of other subunits, the α_1 subunits alone are functional. The specification also clearly states on page 7 that the subunits are by themselves T-type calcium channels and teaches the person of ordinary skill in the art how to make recombinant cells utilized in the claimed methods (*see e.g.,* page 16, lines 19-27). The specification also teaches methods of using such receptors and recombinant cells in standard methods for screening agonists and antagonists. These well-established methods include whole patch clamp analysis, single channel analysis, ^{45}Ca uptake, fluorescence spectroscopy using calcium sensitive dyes such as FURA-2, and binding or displacement of radiolabeled ligands that interact with the calcium channel (*see e.g.,* page 22, lines 4-9). The Office recognized that the specification teaches these uses of the claimed processes as it admitted in the action mailed July 2, 2002 on page 7 that the specification teaches cell lines expressing α_1 subunits can be used

to evaluate the effects of pharmaceuticals and/or toxic substance on calcium channels. As the specification also teaches that the calcium channel subunit may be associated with human genetic diseases, the logical conclusion is the agonists and antagonists identified by the claimed screening assays are expected to be useful for treating these diseases. These diseases include including epilepsy, migraine, ataxia, schizophrenia, hypertension, arrhythmia, angina, depression, small lung carcinoma, Lambert-Eaton syndrome, and Parkinson's disease (*see* specification at page 9). Thus, the specification teaches the claimed processes are useful for identifying agonists and antagonists of T-type calcium channels and diseases associated with such channels.

Second, journal articles published before and at the time the priority application was filed in February of 1997 show that T-type calcium channel agonists were useful for treating a number of diseases set forth in the specification on page 9. These publications are exemplified by the abstracts attached herewith as Exhibits A through H. Abstracts in Exhibits A, B, C, D, E and F show the utility of the T-type calcium channel antagonist mibefradil for treating hypertension, heart disease, and stroke. The abstract in Exhibit G shows the utility of T-type calcium channel blockers nickel and amiloride for affecting the pathogenesis of insulinoma tumor cells. The abstract in Exhibit H shows the utility of the T-type calcium channel blocker zonisamide for treating epileptic seizures. Thus, the utility of T-type calcium channel screening assays for identifying therapeutic molecules was well-established before the priority application was filed.

Third, a compound that binds to one T-type calcium channel binds to all T-type calcium channels. Therefore a compound that treats a disease by binding one T-type channel will bind another T-type calcium channel and exert an effect due to the sequence homology between these channels. As it is well-established there are known T-type calcium channel antagonists useful for treating diseases listed in the specification, the claimed methods have utility for identifying therapeutic molecules. These facts are set forth in the declaration of Dr. Terrence Snutch executed July 10, 2001 and attached herewith as Exhibit I. The Office must consider the factual

analysis by Dr. Snutch as required by the utility examination guidelines (*Official Gazette*, January 30, 2001):

> Office personnel are reminded that they must treat as true a statement of fact made by an applicant in relation to an asserted utility, unless countervailing evidence can be provided that shows that one of ordinary skill in the art would have a legitimate basis to doubt the credibility of such a statement. Similarly, Office personnel must accept an opinion from a qualified expert that is based upon relevant facts whose accuracy is not being questioned; it is improper to disregard the opinion solely because of a disagreement over the significance or meaning of the facts offered.

Because there is no evidence on the record refuting these facts, they must be considered as being true. Thus, the link between the claimed screening assays and the utility of agonists identified by the assays for treating T-type channel-associated diseases is clear.

Fourth, the claimed screening assays have a well-established, specific, substantial and credible utility. The utility is specific because the claimed screening assays are useful for identifying agonists and antagonists of the specified α_1 subunits. The utility is substantial as the agonists and antagonists identified by the screening methods have the real world use of treating diseases specified on page 9 of the specification. The utility is credible because the applicants have generated recombinant cells according to the claimed specification and have screened multiple molecules that act as agonists or antagonists of the α_1 T-subunits (a declaration demonstrating such experiments can be provided to the Office if required). The utility also is credible because the publications submitted in Exhibits A though H demonstrate the usefulness of particular T-type calcium channel agonists for treating diseases. Further, the utility of the claimed screening methods is well-established as evidenced by the state of the art before the filing date of the present patent application. For example, the patch clamp assays and radiolabel ligand assays referenced in the specification on page 22 were well-known in the art as of 1992. *See e.g.,* Williams *et al., Science* 257: 389-395 (1992) and Williams *et al., Neuron*: 71-84 (1992), attached herewith as Exhibits J and K, respectively. Also, the publications in Exhibits A though H show the utility of T-type calcium channel screening assays for identifying agonists useful

from treating certain diseases. Accordingly, the claimed processes have a well-established, specific, substantial and credible utility.

Fifth, the claimed processes require little if no further experimentation for identifying agonists and antagonists useful for treating T-type channel-associated diseases. This feature is highlighted in the Snutch declaration discussed above (Exhibit I) in by journal articles published at the time and before the priority application was filed in 1997 (Exhibits A through H). As the use of the specified α_1 subunits in the claimed screening methods directly identify compounds useful for treating the diseases set forth in the specification, the claimed subject matter is not merely a hunting license as compared to the technology at issue in *Brenner v. Manson*. Also, there is no requirement to determine whether new agonists and antagonists identified by the claimed assays in fact treat a disease *in vivo* in accordance with *In re Brana*, 34 USPQ.2d 1436 (Fed. Cir. 1995). Accordingly, there is little if no further experimentation required to ascertain whether the agonists or antagonists identified by the claimed assays are useful for treating diseases associated with aberrations in α_1 subunits.

Sixth, the Office appears to have ignored the precedence of at least two patents have issued directed to nucleotide sequences encoding T-type calcium channels. These patent are U.S. Patent Nos. 6,358,706 and 6,309,858, attached herewith as Exhibits L and M, respectively. Based on the issuance of these patents the T-type calcium channels clearly are useful as presently claimed. Specifically, they are useful for identifying agonists and antagonists of calcium channel activity which in turn are useful for treating a number of conditions. These utilities are exactly the same as those stated by the patentees in these issued patents. *See e.g.,* columns 6, lines 33-50 in the '706 patent and column 19, lines 53-57 of the '858 patent. In addition, the Office has issued an entire litany of patents with respect to N-type calcium ion channels based on the same logic that applicants have been asserting in the present application. For example, U.S. Patent No. 6,096,514; U.S. Patent No. 6,090,626; U.S. Patent No. 6,013,474; and U.S. Patent No. 5,876,958 issued with the same requisite utility established for the presently claimed screening assays.

Accordingly, the claimed processes have a well-established, specific, substantial, credible and utilities as taught in the specification. As such, the applicants respectfully request that the Office withdraw the rejections under 35 U.S.C. §§ 101 and 112, first paragraph.

CONCLUSION

Given that the Office switched the species election requirement to a restriction requirement in the previous Office action, the applicants respectfully request that the Office consider the arguments set forth herein which demonstrate that the requirement for restriction is improper. The applicants also respectfully submit that the claimed screening methods have a specific, substantial, credible and well-established utility, and therefore, it is respectfully requested that the Office withdraw the rejections under 35 U.S.C. §§ 101 and 112, first paragraph.

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, applicants petition for any required relief including extensions of time and authorize the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket No. 381092000720.

Respectfully submitted,

Dated: November 5, 2003 By: _____

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Am J Hypertens. 1997 Feb;10(2):189-96.

The antihypertensive efficacy of the novel calcium antagonist mibefradil in comparison with nifedipine GITS in moderate to severe hypertensives with ambulatory hypertension.

Lacourciere Y, Poirier L, Lefebvre J, Archambault F, Dalle Ave S, Ward C, Lindberg E.

Hypertension Unit, le Centre Hospitalier de l'Universite Laval, Saint-Foy, Quebec, Canada.

Mibefradil is a novel calcium antagonist that blocks selectively the T-type calcium channels. In this double-blind forced titration study design we compared the effects of mibefradil 50, 100, and 150 mg and nifedipine GITS 30, 60, and 90 mg monotherapies or combined with lisinopril 20 mg in 71 moderate to severe hypertensives (59 men and 12 women) with confirmed ambulatory hypertension. An incremental dose-response effect was observed both in clinic and ambulatory blood pressure parameters during treatment with mibefradil and nifedipine GITS alone and combined with lisinopril. At maximal dosage, patients treated with mibefradil experienced a greater ($P < .05$) reduction in clinic and ambulatory diastolic blood pressures as well as a greater response rate (86% v 69%). Trough:peak ratios for systolic and diastolic blood pressures were > 90% at each dose level. Significant decrease in baseline heart rate was observed with mibefradil 150 mg alone or combined with lisinopril, but no patients experienced clinically significant atrioventricular conduction abnormalities. Adverse events related to vasodilation were more prevalent in the nifedipine GITS group. Consequently, the results of the present study demonstrate that the novel calcium channel blocker mibefradil, either alone or in combination with lisinopril, is effective in reducing clinic and 24-h blood pressures while decreasing heart rate and is well tolerated in patients with moderate to severe hypertension.

Publication Types:

- Clinical Trial
- Randomized Controlled Trial

PMID: 9037327 [PubMed - indexed for MEDLINE]

Clin Ther. 1996 Nov-Dec;18(6):1191-206.

Evaluating the safety of mibefradil, a selective T-type calcium antagonist, in patients with chronic congestive heart failure.

van der Vring JA, Bernink PJ, van der Wall EE, van Velhuisen DJ, Braun S, Kobrin I.

Martini Ziekenhuis, Groningen, The Netherlands.

Mibefradil is a novel calcium antagonist belonging to a new chemical class of benzimidazolyl-substituted tetraline derivatives. The safety of mibefradil in patients with mild-to-moderate chronic congestive heart failure (CHF) due to coronary heart disease was assessed in a randomized, double-masked, placebo-controlled, multiple-ascending-dose trial in 45 patients. Patients were assigned to receive one of five dose levels (6.25, 12.5, 25, 50, or 100 mg/d) of mibefradil or placebo according to a randomization list. If safety variables remained stable, the subsequent group of patients was randomized to the next higher dose. The safety variables assessed included New York Heart Association class, vital signs, and ejection fraction. Patients were evaluated at baseline and day 8 of the dosing period. Mibefradil did not worsen clinical or cardiac variables. Approximately 23.3% (7 of 30) of the mibefradil-treated patients reported one or more adverse events compared with 13.3% (2 of 15) of the placebo group. The incidence of adverse events was not dose dependent. In summary, short-term oral dosing of mibefradil did not worsen measures of cardiac function in 30 patients with mild-to-moderate CHF.

Publication Types:

- Clinical Trial
- Multicenter Study
- Randomized Controlled Trial

PMID: 9001836 [PubMed - indexed for MEDLINE]

J Am Coll Cardiol. 1996 Oct;28(4):972-9.

Hemodynamic and cardiac effects of the selective T-type and L-type calcium channel blocking agent mibefradil in patients with varying degrees of left ventricular systolic dysfunction.

Rousseau MF, Hayashida W, van Eyll C, Hess OM, Benedict CR, Ahn S, Chapelle F, Kobrin I, Pouleur H.

Division of Cardiology, University of Louvain, Brussels, Belgium.

OBJECTIVES: This study sought to assess the hemodynamic and cardiac effects of two dose levels of mibefradil in patients with varying degrees of ischemic left ventricular dysfunction. BACKGROUND: Mibefradil is a new, selective T-type and L-type calcium channel blocking agent. Because L-type channel blockade may depress myocardial performance, an invasive hemodynamic study was performed to assess the safety of this agent. METHODS: We performed an open label study, examining the effects of two intravenous doses of mibefradil, selected to produce plasma levels comparable to those measured after oral administration of 50 mg (dose 1: 400 ng/ml) or 100 mg (dose 2: 800 ng/ml) of the drug. Variables studied included the indexes of left ventricular function and neurohormone levels. Patients were stratified according to ejection fraction (EF) (> or = 40%, n = 26; < 40%, n = 24) and the presence (n = 15) or absence (n = 35) of heart failure. RESULTS: In patients with preserved systolic function, dose 1 had no clinically significant hemodynamic effects, but dose 2 decreased mean aortic pressure and systemic vascular resistance (-8.5 mm Hg, -12%, both $p < 0.01$) and also reduced end-systolic stress and volume, thus improving EF (52% to 58%, $p < 0.01$). Heart rate tended to decrease. In patients with depressed EF, heart rate decreased significantly with both doses. The effects of dose 1 mimicked those observed after dose 2 in patients with preserved EF. Dose 2 (plasma levels 1,052 +/- 284 ng/ml) still decreased left ventricular systolic wall stress and improved EF (24.0% to 28.5%, $p < 0.05$) but also significantly depressed the maximal first derivative of left ventricular pressure. Examination of individual pressure-volume loops in two patients with heart failure showed a clear rightward shift of the loop despite a decrease in systolic pressure, suggesting negative inotropy. Neurohormone levels were unchanged at both dose levels and in all subgroups. CONCLUSIONS: Intravenous mibefradil was well tolerated and produced an overall favorable cardiovascular response. However, high plasma concentrations might produce myocardial depression in patients with heart failure, and caution should be exerted in this setting.

Publication Types:

- Clinical Trial
- Multicenter Study

PMID: 8837576 [PubMed - indexed for MEDLINE]

J Cardiovasc Pharmacol. 1996 Aug;28(2):271-7.

Effects of mibefradil on large and small coronary arteries in conscious dogs: role of vascular endothelium.

Karila-Cohen D, Dubois-Rande JL, Giudicelli JF, Berdeaux A.

Department de Pharmacologie, Faculte de Medecine Paris-Sud, France.

The systemic and coronary hemodynamic effects of mibefradil, a "nondihydropyridine" calcium antagonist acting on both L- and T-type calcium channels, were investigated in chronically instrumented conscious dogs before and after local endothelium removal of the circumflex coronary artery by angioplasty. After intravenous infusion, mibefradil (0.2 mg kg-1 min-1) decreased mean arterial blood pressure (MAP; -15 +/- 1%), increased heart rate (HR; 58 +/- 9%), and coronary blood flow (CBF; 103 +/- 14%) (all $p < 0.05$). Before endothelium removal, mibefradil increased the diameter of the left circumflex epicardial coronary artery (LCX) by 7.8 +/- 1.2% from 3,006 +/- 219 microns, but this dilatory effect was significantly reduced by 69% ($p < 0.001$) and 45% ($p < 0.01$), 3 and 21 days after endothelium removal, respectively. Mibefradil also reduced by 46% ($p < 0.01$) the potent coronary constrictor effect of ergonovine (300 micrograms intravenous bolus). These results demonstrate that mibefradil is a potent dilator of large and small coronary arteries in conscious dogs and that approximately 30% of its dilatory effect on large coronary artery is endothelium-independent. In addition, mibefradil prevents ergonovine-induced epicardial coronary constriction.

PMID: 8856484 [PubMed - indexed for MEDLINE]

J Cardiovasc Pharmacol. 1996 May;27(5):686-94.

Mibefradil, a selective calcium T-channel blocker, in stroke-prone spontaneously hypertensive rats.

Vacher E, Richer C, Fornes P, Clozel JP, Giudicelli.

Departement de Pharmacologie, Faculte de Medecine Paris-Sud, Le Kremlin-Bicetre, France.

Several types of antihypertensive agents, including calcium antagonists, have been reported to prevent stroke and prolong survival in stroke-prone spontaneously hypertensive rats (SHR-SP). We investigated whether mibefradil, a new calcium antagonist acting selectively at the level of T-type calcium channels, would be able to (a) limit or prevent the structural and functional alterations that develop in the cerebral arteries of SHR-SP before stroke and (b) suppress stroke and prolong survival. Mibefradil (30 mg/kg/day) was given orally to young salt-loaded SHR-SP from age 5 weeks to age 20 weeks. Blood pressure (BP) (in conscious animals), diuresis, and proteinuria were determined weekly. After 1012 weeks of treatment, middle cerebral arteries and aortas were removed from randomly selected control and treated SHR-SP. Aortic media thickness and collagen density were evaluated by histomorphometry. Middle cerebral arteries were mounted in a myograph for wall thickness determination and isometric tension recordings. Mibefradil completely prevented stroke and mortality, significantly limited the increase in BP, and opposed the increases in diuresis and proteinuria observed in controls. Simultaneously, mibefradil abolished vascular fibrinoid necrosis formation in the brain and reduced arterial thickening in the cerebral artery as well as in the aorta. The maximal contractile responses of the cerebral arteries to potassium chloride and serotonin were greater in mibefradil-treated animals than in controls, as were the endothelium-dependent relaxant responses. Mibefradil, chronically administered to young SHRSP in a dose that limits the development of hypertension not only prevents stroke and mortality but also affords protection against the vascular structural alterations which develop with age in these animals and preserves or improves the cerebral artery's smooth muscle and endothelial cell functions.

PMID: 8859939 [PubMed - indexed for MEDLINE]

Cardiovasc Drugs Ther. 1996 May;10(2):101-5.

Prevention of neointima formation by mibefradil after vascular injury in rats: comparison with ACE inhibition.

Schmitt R, Clozel JP, Iberg N, Buhler FR.

Pharma Division, F. Hoffmann-La Roche Ltd., Basel, Switzerland.

Cilazapril, an angiotensin-converting enzyme inhibitor, and mibefradil, a selective T-type voltage-operated calcium channel blocker, have been shown to prevent neointima formation after vascular injury. The goal of the present study was to evaluate the mechanism of action of both drugs. For this purpose, the influence of the renin angiotensin system on the effects of mibefradil (30 mg/kg po) and cilazapril (10 mg/kg po) on neointima formation after carotid injury were evaluated in normotensive rats (normal renin angiotensin system) and DOCA hypertensive rats (suppressed renin angiotensin system). In addition, in order to differentiate an effect on cell migration or cell proliferation, both drugs were given either before or after the smooth muscle migration phase. Finally, cilazapril and mibefradil were given in combination. In normotensive rats, mibefradil and cilazapril decreased neointima formation, resulting in neointima/media ratios of 38% ($p < 0.05$) and 53% ($p < 0.01$), respectively. However, in DOCA hypertensive rats, mibefradil was active, with a reduction of the neointima/media ratio by 63% ($p < 0.001$), whereas cilazapril reduced it only slightly (19%) and not significantly. In addition, cilazapril was active only when treatment started before the migration phase (63%, reduction in neointima/media ratio, $p < 0.001$) but not when started thereafter (13% reduction in neointima/media ratio, n.s.). In contrast, treatment with mibefradil was also active when started after the migration phase (51% reduction in neointima/ media ratio, $p < 0.001$ when treatment started 1 day before balloon injury and 41%, $p < 0.01$ when treatment started 5 days after balloon injury). The combination of both drugs was additive (67% reduction in neointima/media ratio, $p < 0.001$ vs. control). These experiments clearly show that mibefradil and cilazapril have a different mechanism of action after vascular injury. Mibefradil most likely prevents the proliferation of smooth muscle cells. In contrast, cilazapril most likely inhibits the migration of smooth muscle cells. These two different mechanisms of action explain why the effects of both drugs are additive.

PMID: 8842500 [PubMed - indexed for MEDLINE]

Diabetes. 1996 Dec;45(12):1678-83.

Abnormally expressed low-voltage-activated calcium channels in beta-cells from NOD mice and a related clonal cell line.

Wang L, Bhattacharjee A, Fu J, Li M.

Department of Pharmacology, University of South Alabama, College of Medicine, Mobile 36688, USA.

A macroscopic low-voltage-activated (LVA) inward current was found in pancreatic beta-cells isolated from NOD mice. However, this current was not present in nondiabetic prone mouse (e.g., Swiss-Webster) pancreatic beta-cells. We performed pharmacological analyses on this current in NOD insulinoma tumor cells (NIT-1). This cell line was developed from pancreatic beta-cells of a transgenic NOD mouse. The sodium-channel blocker, tetrodotoxin (TTX; 2 micromol/l) had no effect on this LVA current. The amplitudes of currents elicited by a -20 mV test pulse retained similarity when the extracellular sodium concentration was increased from 0 to 115 mmol/l; when the extracellular calcium concentration was decreased from 10 to 2 mmol/l, there was an approximate 50% reduction of this current elicited by a -30 mV test pulse. Neither the L-type calcium-channel blocker, nifedipine (3 micromol/l), nor the N-type calcium-channel blocker, omega-CgTx-GVIA (1 micromol/l), at -30 mV produced an appreciable effect. The T-type calcium-channel blockers, nickel (3 micromol/l) and amiloride (250 micromol/l), effectively reduced the peak of this current. In 2 mmol/l calcium external solution, the threshold of voltage-dependent activation of this calcium current was approximately -65 mV, and the peak current occurred at -20 mV. Half-maximum steady-state inactivation was around -43 mV. The mean time constant of slow deactivating tail currents generated by a preceding 20 mV pulse was 2.53 ms. The intracellular free calcium concentration was two- to threefold higher in NOD mouse pancreatic beta-cells compared with Swiss-Webster pancreatic beta-cells. We concluded that there are LVA calcium channels abnormally expressed in NOD mouse beta-cells. This LVA calcium channel may be factorial to the high cytosolic free calcium concentration observed in these cells, and thereby may contribute to the pathogenesis of NOD mouse beta-cells.

PMID: 8922351 [PubMed - indexed for MEDLINE]

Seizure. 1996 Jun;5(2):115-9.

Mechanisms of T-type calcium channel blockade by zonisamide.

Kito M, Maehara M, Watanabe K.

Department of Pediatrics, Minami Seikyo Hospital, Nagoya, Japan.

We investigated the effects of zonisamide, a new antiepileptic drug, on voltage-dependent T-type calcium current (ICa) in cultured neuroblastoma cells of human origin (NB-I). Zonisamide reduced T-type ICa in a concentration-dependent manner without evoking any change in its inactivation kinetics or voltage dependence of action. The mean percent reduction was 38.3 +/- 5.8% at 50 microM. Further, zonisamide shifted the inactivation curve approximately 20 mV negative compared to the control. These resting blocking actions suggest that zonisamide shifts the channel population toward the inactivation state, allowing fewer channels to open during membrane depolarization. The blockade of T-type calcium channels by zonisamide could suppress an important component of inward current that underlies epileptiform cellular bursting, thereby inhibiting the spread of seizure activity.

PMID: 8795126 [PubMed - indexed for MEDLINE]

PATENT
Docket No. 381092000720

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I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on July 18, 2001.

Tami M Procopio

Tami M. Procopio

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Terrance P. SNUTCH, *et al.*

Serial No.: 09/346,794

Filing Date: 02 July 1999

For: NOVEL HUMAN CALCIUM CHANNELS AND RELATED PROBES, CELL LINES AND METHODS

Examiner: Nirmal S. Basi

Group Art Unit: 1646

DECLARATION OF DR. TERRANCE SNUTCH

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Terrance Snutch, declare as follows:

1. I am a co-inventor of the subject matter claimed in the above-referenced application and have been practicing in the field of molecular biology, and specifically in the field of ion channels, for over 15 years. A copy of my *curriculum vitae* is attached hereto as Exhibit A. I have published many papers on the structure and function of calcium channels and am considered one of the leading researchers in this field.

2. The association of abnormal T-type calcium channel activity with specific conditions is well known in the art. Enclosed herewith are a number of documents which verify this. Abnormal T-type activity is associated with a number of cardiac conditions including

pacemaker activity (Hajiwara, *et al., J. Physiol.* (1988) 395:233-253; cardiac hypertrophy (Nuss, *et al., Circ. Res.* (1995) 73:777-782); and hypertension (Self, *et al., J. Vasc. Res.* (1994) 31:359-366). Abnormal T-type calcium function is also associated with neurological diseases wherein neuronal bursts are abnormally fired causing spastic convulsions (Huguenard, *Ann. Rev. Physiol.* (1996) 58:329-348) and thus associated with epilepsy (Tsakiridou, *et al., J. Neuro. Sci.* (1995) 15:3110-3117; Coulter, *et al., Brit. J. Pharmacol.* (1990) 100:800-806). Abnormal function of the T-type calcium ion channel is also associated with impaired fertility because of its effect on hormone secretion (Rossier, *et al., Endocrinology* (1966) 137:4817-4826; Arnoult, *et al., Proc. Natl. Acad. Sci. USA* (1996) 93:13004-13009). Copies of these documents are attached hereto. Thus the conditions associated with abnormal T-calcium channel function are well established and agonists and antagonists of T-type calcium channels are useful in treating these conditions.

3. There are several T-type calcium channels found in a single individual which vary slightly in structure and demonstrably in terms of their distribution among various tissues. This, however, does not affect the usefulness of screening assays for agonists and antagonists. The particular T-type calcium channel involved in a particular condition may depend on its tissue distribution; for instance, T-type channels found in the nervous system are associated with epilepsy and neurological diseases in general where spastic convulsions are involved. However, it is not necessary to understand which particular T-type calcium channel is being used in a screen for compounds that would be useful in treating, for example, these convulsive conditions because of the similarity in the binding specificity of all T-type channels. In very simple terms, compounds which are found to inhibit or stimulate the activity of nervous T-type channels will also inhibit or stimulate the activity of T-type channels found in other tissues. Thus, any arbitrarily chosen T-type channel could be expressed in a cell line for use in screening assays to identify agonists or antagonists and the agonists or antagonists would be useful in treating the conditions associated with any T-type channel. As noted above, abnormal T-type activity is associated with a number of cardiac conditions, with hypertension, with neurological diseases involving spastic convulsions, and with impaired fertility. An agonist or antagonist identified with regard to any T-type channel would be useful in any and all of these conditions.

4. This pattern of similar binding activity among all T-type channels can be analogized to such a pattern among L-type channels. All of the T-type channels have similar

behaviors in that they activate at low membrane potential, have small single channel conductance, have negative steady state inactivation properties, and contribute to spike firing patterns and rhythmic bursting processes. Analogous to the T-type channel another type of channel linked by similar behaviors is the L-type. There are several α_1 subunits associated with various L-type channels - *i.e.*, α_{1S}, α_{1C}, and α_{1D} and each is encoded by a distinct gene and exhibits a distinct distribution pattern. For example, α_{1S} is in skeletal muscle; α_{1C} is in neurons and cardiac and smooth muscle; and α_{1D} is found in neurons and endocrine cells. They can be discriminated from all other types of calcium channels by their common sensitivity to 1,4-dihydropyridines. Thus, any one of these genes could be used to generate an L-type calcium channel for use in a cell-based assay to identify interacting compounds. These interacting compounds bind to all L-type channels and thus are useful in treating conditions related to any one of them.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed at VANCOUVER, B.C. on 10 July 2001.

Terrance Snutch

concentrations of L-Arg (5 to 100 mM) to muscles treated with L-NMMA and TNF-α resulted in a more pronounced negative inotropic effect than that seen with TNF-α alone [19 ± 4% of baseline tension with TNF-α and L-Arg as compared to 59 ± 7% of baseline tension with TNF-α alone ($P < 0.01$, $n = 6$; Student's two-tailed t test)] (Fig. 4A). This suggests that L-Arg enhanced the negative inotropic effect of TNF-α by providing additional substrate for NO production. This effect was also greater than that seen with L-Arg (100 mM) alone (31 ± 6% of baseline tension; $P < 0.01$, $n = 6$; Student's two-tailed t test). The addition of L-Arg (100 mM) to muscles treated with L-NMMA and IL-6 reduced tension to 35 ± 3% (Fig. 4B). The addition of L-Arg (100 mM) to muscles treated with L-NMMA and IL-2 reduced tension to 11 ± 10% of baseline (Fig. 4C). All of these inotropic effects were completely reversed within 30 min after the cytokines or other agents were washed away (Fig. 4, A through C). Removal of the endothelium did not alter the negative inotropic responses of the papillary muscles to cytokines (Fig. 4, A through C).

Cytokines increase the amount of NO in noncardiac tissues by inducing the transcription of an inducible NO synthase (*13–16*). The rapid onset and reversibility of the effects seen in this report argue against an effect requiring gene transcription. The negative inotropic effects of these cytokines in the papillary muscle preparation appear to result from enhanced activity of a constitutive NO synthase enzyme in the myocardium.

The observed inotropic effects of pro-inflammatory cytokines raise the possibility that they participate in reversible, postischemic myocardial depression ("stunning"). Myocardial stunning frequently occurs after cardiopulmonary bypass and may complicate successful recovery from cardiac surgery (*5–9*). We found elevated concentrations of IL-6 (1800 to 4000 U/ml) in bronchoalveolar fluid from patients after cardiopulmonary bypass (*18*). IL-6 also reversibly decreased tension generated by pectinate muscles removed from patients at the time of surgery (*18*). These preliminary observations in patients support the clinical relevance of our findings with the Syrian hamster papillary muscle preparation. Thus, the regulation of pro-inflammatory cytokines and myocardial NO synthase may provide new therapeutic strategies for the management of cardiac patients.

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Structure and Functional Expression of an ω-Conotoxin–Sensitive Human N-Type Calcium Channel

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N-type calcium channels are ω-conotoxin (ω-CgTx)–sensitive, voltage-dependent ion channels involved in the control of neurotransmitter release from neurons. Multiple subtypes of voltage-dependent calcium channel complexes exist, and it is the α_1 subunit of the complex that forms the pore through which calcium enters the cell. The primary structures of human neuronal calcium channel α_{1B} subunits were deduced by the characterization of overlapping complementary DNAs. Two forms ($\alpha_{1B\text{-}1}$ and $\alpha_{1B\text{-}2}$) were identified in human neuroblastoma (IMR32) cells and in the central nervous system, but not in skeletal muscle or aorta tissues. The $\alpha_{1B\text{-}1}$ subunit directs the recombinant expression of N-type calcium channel activity when it is transiently co-expressed with human neuronal β_2 and α_{2b} subunits in mammalian HEK293 cells. The recombinant channel was irreversibly blocked by ω-CgTx but was insensitive to dihydropyridines. The $\alpha_{1B\text{-}1}\alpha_{2b}\beta_2$-transfected cells displayed a single class of saturable, high-affinity (dissociation constant = 55 pM) ω-CgTx binding sites. Co-expression of the β_2 subunit was necessary for N-type channel activity, whereas the α_{2b} subunit appeared to modulate the expression of the channel. The heterogeneity of α_{1B} subunits, along with the heterogeneity of α_2 and β subunits, is consistent with multiple, biophysically distinct N-type calcium channels.

Voltage-dependent Ca^{2+} channels are multisubunit complexes through which extracellular Ca^{2+} enters excitable cells. In rabbit skeletal muscle, four tightly coupled subunits, α_1, α_2, β, and γ, make up the channel complex (*1*). The primary structure of each subunit has been determined and α_1, α_2, and β cDNAs have been used to characterize transcripts expressed in other tissues (*2*). The α_1 and β subunits are each encoded by a gene family, including at least five distinct genes for α_1 subunits and three genes for β subunits (*3–6*). Primary transcripts of each of the α_1 genes, the α_2 gene, and two of the β genes have been shown to yield multiple, structurally distinct, subunits by means of differential processing (*6–9*). Expression studies have shown that the α_1 subunit forms the pore through which Ca^{2+} enters the cell (*10, 11*).

On the basis of biophysical and pharmacological characteristics, three subtypes of neuronal, high-voltage–activated Ca^{2+} channels (L-, N-, and P-type) have been proposed (*2*). These high-voltage–activated

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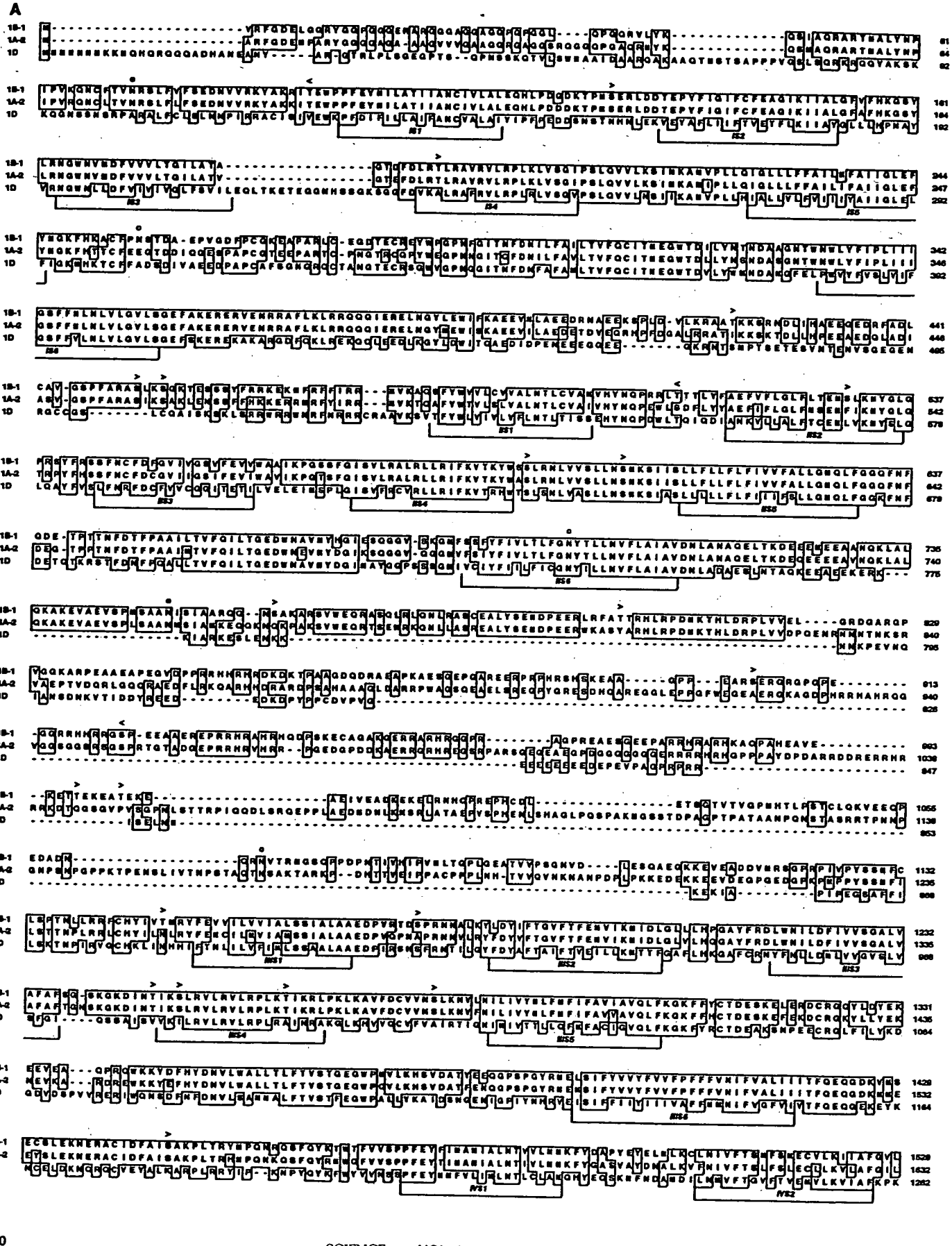
A
1B-1
1A-2
1D

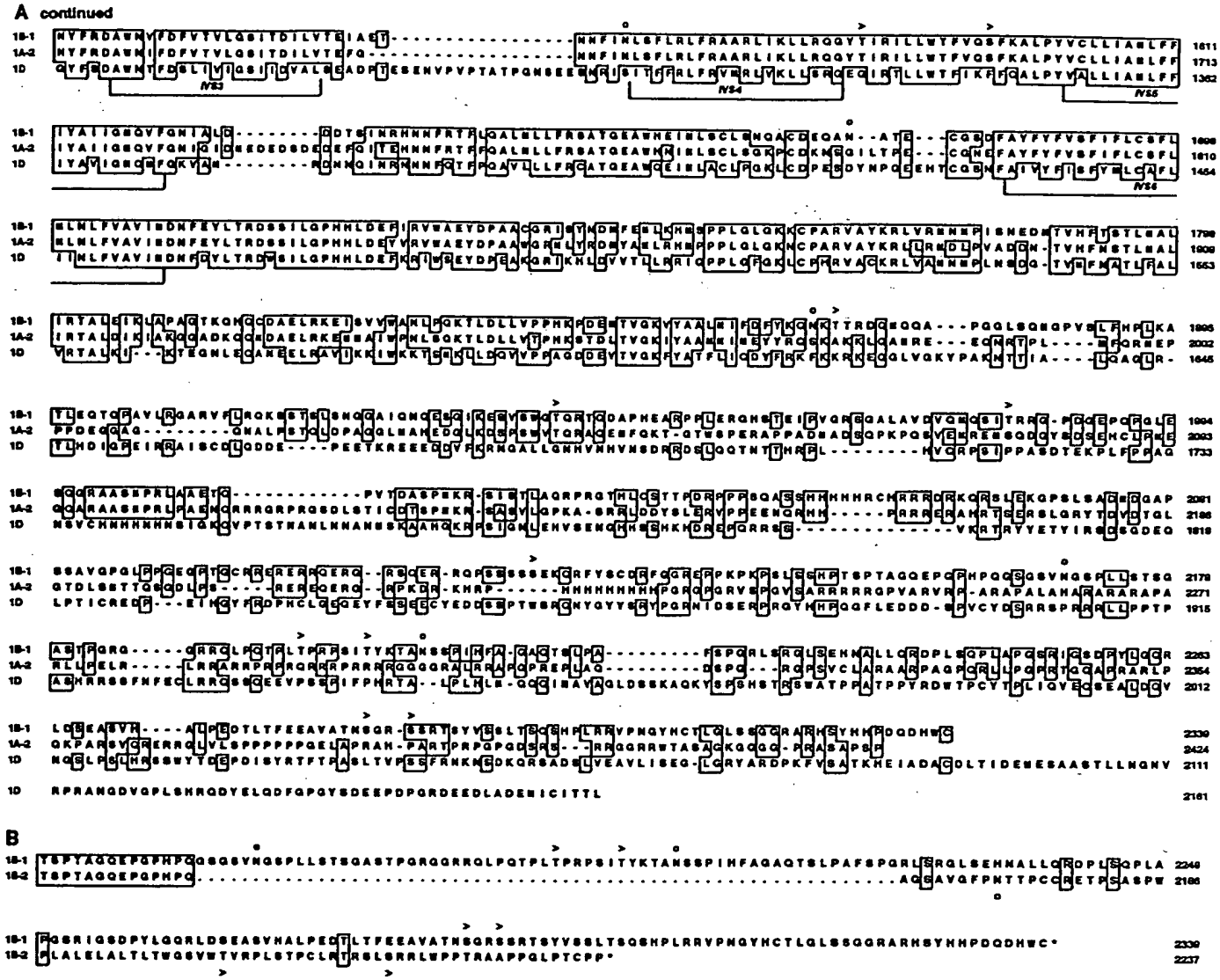

Fig. 1. Alignment of α_1 subunit deduced amino acid sequences. The nucleotide sequences have been deposited in GenBank (accession numbers M94172 and M94173 for α_{1B-1} and α_{1B-2}, respectively). The number of the amino acid residue at the end of each line is given. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. Identical residues at one position in at least two of the sequences are enclosed in boxes. Potential *N*-glycosylation (o), cyclic adenosine monophosphate (AMP)–dependent phosphorylation (<), and protein kinase C phosphorylation (>) sites (*36*) are shown. (**A**) Alignment of functional, neuronal α_1 subunits; the amino acid sequences of the human neuronal Ca^{2+} channel α_{1B-1} (1B-1), the human neuronal α_{1D} (1D) (*8*), and the rabbit brain BI-2 (1A-2) (*11*) are shown. BI-2 is designated 1A-2 because it is a rabbit homolog of the rat brain class A gene (*4*). The numbering begins with the proposed initiating methionine. The putative transmembrane segments S1 through S6 in each of the repeats I through IV are shown (brackets). (**B**) Alignment of α_{1B-1} and α_{1B-2} sequences through the region of the insertion-deletion (*21*). The deduced amino acid sequence of the 187-nt insertion (α_{1B-1} nt 6490 to 6676; Gly^{2164} to Gly^{2226}) is shown.

subtypes are most readily distinguished pharmacologically. The neuronal L-type channel is dihydropyridine (DHP)-sensitive and, in some cases, reversibly blocked by ω-conotoxin (ω-CgTx) (*12*, *13*), the N-type channel is DHP-insensitive and irreversibly blocked by ω-CgTx (*14*), and the P-type channel is both DHP- and ω-CgTx–insensitive but is sensitive to toxins in venom from funnel web spiders (*15*). Recently, recombinant expression of neuronal Ca^{2+} channels was used to identify a high-voltage–activated, DHP-sensitive Ca^{2+} channel that was reversibly blocked by ω-CgTx (classified as an L-type channel) (*8*) and a DHP-, ω-CgTx–insensitive Ca^{2+} channel (possibly a P-type channel) (*11*). Co-expression of α_1 and β subunits is necessary for substantial functional expression of both Ca^{2+} channel subtypes, whereas addition of an α_2 subunit increases the magnitude of the functional response.

Much evidence indicates that DHP-insensitive N-type Ca^{2+} channels that are irreversibly blocked by ω-CgTx are responsible for the voltage-activated release of neurotransmitters in many neurons (*16*). In addition, ω-CgTx binding sites have been localized to the frog neuromuscular presynaptic membrane (*17*) and to organized, single clusters coincident with synaptic contact sites in hippocampal neurons (*18*). Furthermore, ω-CgTx binding sites on the presynaptic membrane of the frog neuromuscular terminal align precisely with active zones where vesicular exocytosis of neurotransmitters occurs (*16*, *17*). Finally, ω-CgTx irreversibly blocks Ca^{2+} currents recorded directly from presynaptic termi-

nals (19). We report here the complete amino acid sequence of a human neuronal α_1 subunit (designated α_{1B}) that mediates N-type voltage-dependent Ca^{2+} channel activity, which is irreversibly blocked by ω-CgTx when transiently co-expressed with the human neuronal α_{2b} and β_2 subunits (8) in human embryonic kidney (HEK) 293 cells. The transfected cells bind ω-CgTx with high affinity.

We previously reported the isolation of cDNAs that encode the α_1 subunit of the rabbit skeletal muscle DHP-sensitive, L-type Ca^{2+} channel (3). These cDNAs were used as probes to isolate overlapping cDNAs encoding a complete human neuronal α_{1B} subunit (20). The translation initiation site was assigned to the first in-frame methionine codon, and no upstream in-frame nonsense codon was identified (Fig. 1A). Two isoforms of α_{1B}, $\alpha_{1B\text{-}1}$ and $\alpha_{1B\text{-}2}$, that differ at their COOH-termini were identified (Fig. 1B). The $\alpha_{1B\text{-}1}$ subunit is comprised of 2339 amino acids and yields a calculated molecular weight of 262,494, whereas the $\alpha_{1B\text{-}2}$ subunit is comprised of 2237 amino acids and yields a calculated molecular weight of 251,757. These isoforms were identified by polymerase chain reaction (PCR) analysis (21) and revealed a deletion that produces $\alpha_{1B\text{-}2}$, which likely results from alternative selection of a splice acceptor. This insertion-deletion that produces different COOH-termini is similar to the processing of putative rabbit α_{1A} gene transcripts encoding the rabbit BI-1 and BI-2 isoforms that mediate DHP-, ω-CgTx–insensitive high-voltage–activated Ca^{2+} channel activity (11). The α_{1B} sequence is 94.5% identical to the previously reported 164–amino acid sequence deduced from a rat brain class B partial cDNA (4) and has the same transmembrane topology as described previously for other Ca^{2+} channel α_1 subunits (7).

The deduced amino acid sequences of two different neuronal α_1 subunits, the human α_{1D} (8) and the rabbit BI-2 (11), are shown aligned with the human $\alpha_{1B\text{-}1}$ sequence (Fig. 1A). The $\alpha_{1B\text{-}1}$ amino acid sequence is 64.1% and 43.0% identical to the BI-2 and α_{1D} sequences, respectively. The sequence identity is relatively well conserved through the four repeating domains, 72.6% and 50.7% for the $\alpha_{1B\text{-}1}$/BI-2 and the $\alpha_{1B\text{-}1}/\alpha_{1D}$ pairs, respectively. Both of the DHP-insensitive α_1 subunits, human neuronal $\alpha_{1B\text{-}1}$ and rabbit neuronal BI-2, have characteristic large putative cytoplasmic loops between the IIS6 and IIIS1 transmembrane domains. PCR analysis performed on RNAs isolated from IMR32 cells and several human primary tissues with $\alpha_{1B\text{-}1}$- and $\alpha_{1B\text{-}2}$-specific oligonucleotides identified $\alpha_{1B\text{-}1}$ and $\alpha_{1B\text{-}2}$ transcripts in IMR32 cells and in each of the human central nervous system (CNS) tissues tested, including hippocampus, habenula, and thalamus but not in human skeletal muscle or aorta tissues (22).

The transient expression of the human neuronal $\alpha_{1B\text{-}1}$, α_{2b}, and β_2 (8) subunits was studied in HEK293 cells (23). Transfected cells were examined for inward Ba^{2+} currents (I_{Ba}) mediated by voltage-dependent Ca^{2+} channels (24). Cells cotransfected with the $\alpha_{1B\text{-}1}$, α_{2b}, and β_2 cDNAs expressed high-voltage–activated Ca^{2+} channels (Fig. 2). I_{Ba} first appeared when the membrane was depolarized from a holding potential of −90 mV to −20 mV and peaked in magnitude at 10 mV. Thirty-nine of 95 cells (12 independent transfections) had I_{Ba} that ranged from 30 to 2700 pA, with a mean of 433 pA. The mean current density was 26 pA/pF, and the highest density was 150 pA/pF (25). The I_{Ba} typically increased by 2- to 20-fold during the first 5 min of recording. Repeated depolarizations during long recordings often revealed rundown of I_{Ba} usually not exceeding 20% within 10 min. I_{Ba} typically activated within 10 ms and inactivated with both a fast time constant ranging from 46 to 105 ms and a slow time constant ranging from 291 to 453 ms ($n = 3$). Inactivation showed a complex voltage dependence, such that I_{Ba} elicited at ≥20 mV inactivated more slowly than I_{Ba} elicited at lower test voltages, possibly a result of an increase in the magnitude of slow compared to fast inactivation components at higher test voltages.

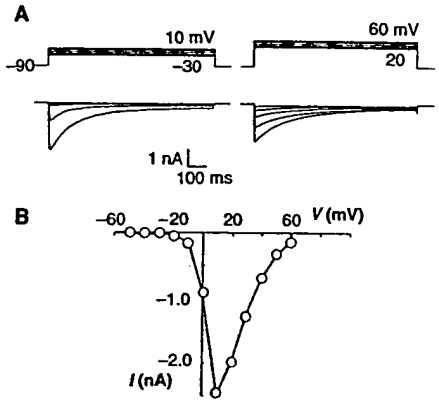

Fig. 2. Voltage dependence and kinetics of I_{Ba} expressed in HEK293 cells transfected with $\alpha_{1B\text{-}1}$, α_{2b}, and β_2 cDNAs (23). (**A**) Family of currents evoked at test voltages from −30 to 60 mV, from a holding potential of −90 mV. (**B**) Peak current-voltage relations measured from the currents in (A).

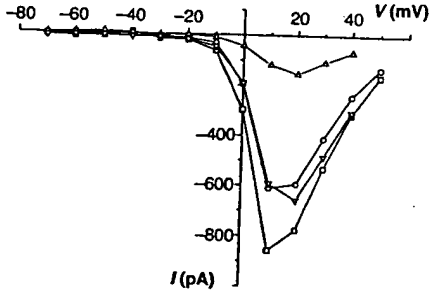

Fig. 3. Holding potential sensitivity of I_{Ba} expressed in HEK293 cells transfected with $\alpha_{1B\text{-}1}$, α_{2b}, and β_2 cDNAs (23). Peak current-voltage (*I-V*) relations measured from voltage steps delivered from different holding potentials (−90 mV, □; −70 mV, ○; −50 mV, △; return to −90 mV, ▽).

Recombinant $\alpha_{1B\text{-}1}\alpha_{2b}\beta_2$ channels were sensitive to holding potential (Fig. 3). Steady-state inactivation of I_{Ba}, measured after a 30- to 60-s conditioning at various holding potentials, was approximately 50% at holding potentials between −60 and −70 mV and approximately 90% at −40 mV. Recovery of I_{Ba} from inactivation was usually incomplete, measuring 55 to 75% of the original magnitude within 1 min after the holding potential was returned to more negative potentials, possibly indicating some rundown or a slow recovery rate.

Recombinant $\alpha_{1B\text{-}1}\alpha_{2b}\beta_2$ channels were also blocked irreversibly by ω-CgTx concentrations ranging from 0.5 to 10 μM during the time scale of the experiments (Fig. 4). Application of 5 μM toxin ($n = 7$) blocked the activity completely within 2 min, and we observed no recovery of I_{Ba} after washing ω-CgTx from the bath for up to 15 min. Cd^{2+} blockage (50 μM) was rapid, complete, and reversible; the DHPs Bay K 8644 (1 μM; $n = 4$) or nifedipine (5 μM; $n = 3$) had no discernable effect.

Cells cotransfected with cDNAs encoding $\alpha_{1B\text{-}1}$, α_{2b}, and β_2 subunits predominantly displayed a single class of saturable, high-affinity ω-CgTx binding sites (26) (Fig. 5). The determined dissociation constant (K_d) value (Fig. 5) was 54.6 ± 14.5 pM ($n = 4$). Cells transfected with the vector containing only β-galactosidase cDNA or $\alpha_{2b}\beta_2$ cDNA showed no specific binding. The binding capacity (B_{max}) of the $\alpha_{1B\text{-}1}\alpha_{2b}\beta_2$-transfected cells was 28,710 ± 11,950 sites per cell ($n = 4$).

These results demonstrate that $\alpha_{1B\text{-}1}$-$\alpha_{2b}\beta_2$-transfected cells express high-voltage–activated, inactivating Ca^{2+} channel activity that is irreversibly blocked by ω-CgTx, insensitive to DHPs, and sensitive to holding potential. The activation and inactivation kinetics and voltage sensitivity of the channel formed in these cells are generally consistent with previous characterizations of neuronal N-type Ca^{2+} channels (27, 28). Furthermore, the K_d value determined for ω-CgTx binding is in agreement with previously reported values (29).

The binding characteristics of ω-CgTx to HEK293 cells transiently expressing various subunit combinations were determined

from saturation binding analysis (Table 1). Each recombinant cell type displayed a single class of binding sites similar to the $\alpha_{1B-1}\alpha_{2b}\beta_2$-transfected cells, with K_d values ranging from 38.8 ± 13.1 pM to 76.1 ± 15.5 pM. The binding affinity of the recombinant cell types for ω-CgTx agrees well with that determined for intact IMR32 cells (36.5 ± 6.2 pM) (Table 1) but is different from measurements derived from crude homogenates of IMR32 cells (*30*).

There were significant differences in the receptor densities of the four recombinant cell types (Table 1). The B_{max} for ω-CgTx binding in $\alpha_{1B-1}\alpha_{2b}\beta_2$-type cells was approximately ten times greater than that in $\alpha_{1B-1}\alpha_{2b}$- and α_{1B-1}-type cells. The estimate for the binding capacity of the IMR32 cells correlates well with a previous report (*30*). The comparison of the B_{max} values suggests that the ω-CgTx–binding α_{1B-1} subunit is more efficiently expressed on the cell surface when co-expressed with the α_{2b} and β_2 subunits. Similarly, efficient expression of heteromeric protein complexes on the cell surface, such as nicotinic acetylcholine receptors, has been shown to require subunit assembly (*31*).

We performed whole cell recordings of HEK293 cells transfected with the cDNA encoding α_{1B-1} or with cDNAs encoding α_{1B-1} and α_{2b} or β_2 to assess functional contributions of the various subunits to the N-type channel activity. Currents recorded from $\alpha_{1B-1}\beta_2$-transfected cells were observed at a frequency comparable to that of the $\alpha_{1B-1}\alpha_{2b}\beta_2$-transfected cells (16 of 46 cells; five independent transfections), consistent with a B_{max} of approximately 12,000 receptors per cell (Table 1). These currents resembled those observed in $\alpha_{1B-1}\alpha_{2b}\beta_2$-transfected cells, having similar current-voltage (*I-V*) curves, inactivation kinetics, and sensitivity to holding potential. Furthermore, $\alpha_{1B-1}\beta_2$-mediated currents were irreversibly blocked by ω-CgTx (5 μM; n = 3). However, currents in $\alpha_{1B-1}\beta_2$-transfected cells were generally smaller in magnitude than those observed in $\alpha_{1B-1}\alpha_{2b}\beta_2$ cells and never exceeded 205 pA (15 pA/pF), with a mean of 91 pA (5.6 pA/pF). In contrast, currents in $\alpha_{1B-1}\alpha_{2b}\beta_2$-transfected cells exceeded 200 pA in 57% of the cells tested (*25*).

Of 23 cells studied that were transfected with only α_{1B-1} (three independent transfections), two had small (20 to 40 pA) rapidly inactivating (τ = ~20 ms) currents that were reversibly blocked by ω-CgTx. A similar current was detected in 1 of 11 $\alpha_{1B-1}\alpha_{2b}$-transfected cells, whereas none of the untransfected HEK293 cells (n = 17) or HEK293 cells transiently expressing the α_{2b} and β_2 subunits (n = 17) displayed such currents. These results together with the relatively small B_{max} values observed in α_{1B-1}-only and $\alpha_{1B-1}\alpha_{2b}$-transfected cells (<2650 receptors per cell) further support the importance of the β subunit in the formation of functional N-type Ca^{2+} channels.

N-type Ca^{2+} channels characterized from different cell preparations have biophysically distinct properties that have made it difficult to distinguish N- and L-type currents on the basis of inactivation properties. N-type Ca^{2+} channels were first described in chicken sensory neurons as high-voltage–activated Ca^{2+} channels that could be activated only from strongly negative holding potentials and inactivated within tens of milliseconds (*27*). Current remaining after decay of the inactivating component or currents activated from holding potentials ≥ −40 mV were believed to represent L-type channel activity. N-type Ca^{2+} channels have since been found to inactivate slowly and incompletely in some neuronal types (*32*). The range of inactivation rates observed in different tissues may be a result of a combination of factors, including distinct combinations of variant channel subunits and different states of regulation. Recent single channel analysis indicates that individual N-type channels can switch between transient and long-lasting modes of gating (*33*). Our whole cell data that show biphasic decay of a recombinantly expressed N-type Ca^{2+} channel are consistent with a population of channels that exhibit different gating modes.

Recent biochemical studies on brain ω-CgTx receptors have revealed proteins on SDS–polyacrylamide gel electrophoresis of a relative molecular mass consistent with α_1, α_2, and β subunits (*29*), although additional uncharacterized bands were also observed. Molecular biological evidence indicates that multiple α_1, α_2, and β transcripts, including α_{1B}, α_{2b}, and β_2 mRNAs, are co-expressed in IMR32 cells and hippocampal tissue (*8*), both sources of ω-CgTx binding sites (*18*, *30*). The recombinant expression of α_{1B-1}, α_{2b}, and β_2 subunits to produce ω-CgTx–sensitive N-type channel activity confirms that an α_{1B} gene product mediates this activity. The functional necessity of a β subunit and modulation by an

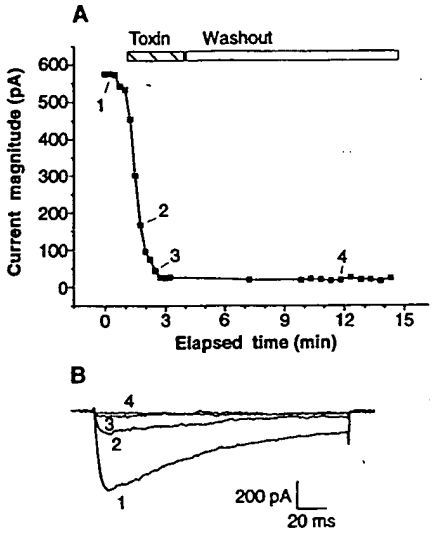

Fig. 4. Effect of ω-CgTx on I_{Ba} expressed in HEK293 cells transfected with α_{1B-1}, α_{2b}, and β_2 cDNAs (*23*). (**A**) Plot of peak current magnitude versus time before, during (hatched bar), and after (open bar) application of 5 μM ω-CgTx. Test pulses (10 mV; holding potential = −90 mV) were delivered every 15 s before and during toxin application. Pulses were resumed every 30 s after recording of current-voltage relations from which only the current measured at 10 mV is shown. Similar results were obtained with the three concentrations of ω-CgTx tested: 0.5 μM (n = 3), 5 μM (n = 7), and 10 μM (n = 6). (**B**) Example recordings made at points 1 to 4 of (A).

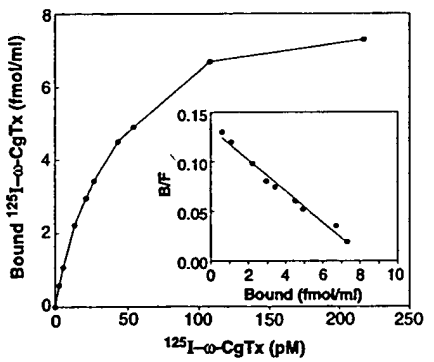

Fig. 5. Binding of ^{125}I–ω-CgTx. HEK293 cells were cotransfected with the α_{1B-1}, α_{2b}, and β_2 cDNAs (*23*) and assayed for specific binding of ^{125}I–ω-CgTx as a function of increasing concentration of ^{125}I–ω-CgTx (*26*); 2 × 10^5 cells were used in the assay mixture. (Inset) Scatchard analysis of the data. B, bound; F, free.

Table 1. Summary of Scatchard analysis of ω-CgTx binding to intact cells. HEK293 cells transfected with the indicated subunit cDNAs and IMR32 cells induced with dibutyryl cyclic AMP and bromodeoxyuridine (*28*) were assayed for saturation of specific ω-CgTx binding, and the data were analyzed by the Scatchard method (*26*). The B_{max} values determined from Scatchard analysis were corrected for transfection efficiency.

Cell line	K_d (pM)	B_{max} (sites/cell)
$\alpha_{1B-1}\alpha_{2b}\beta_2$	54.6 ± 14.5	28,710 ± 11,950 (n = 4)
$\alpha_{1B-1}\beta_2$	38.8 ± 13.1	11,860 ± 5,910 (n = 4)
$\alpha_{1B-1}\alpha_{2b}$	76.1 ± 15.5	2,650 ± 620 (n = 4)
α_{1B-1}	59.1 ± 15.5	2,085 ± 880 (n = 4)
IMR32	36.5 ± 6.2	6,770 ± 615 (n = 2)

α_2 subunit are consistent with the recombinant functional expression of other α_1 subtypes (*8*, *11*), although expression of $\alpha_{1B\text{-}1}$ alone appears sufficient for ω-CgTx binding.

Our results suggest that multiple subtypes of the N-type channel might exist as a result of the heterogeneity of the subunits that comprise the channel complex. Co-expression of three different β gene products with the rabbit cardiac (α_{1C}) subunit alters the channel properties and thus indicates that subunit composition can determine distinct, voltage-dependent Ca^{2+} channels (*6*). At least two forms each of α_{1B}, α_2, and β transcripts expressed in the brain are products of differential processing (*6*, *8*, *34*). This heterogeneity of the α_{1B}, α_2, and β subunits is consistent with biophysically distinct N-type channels characterized from different cell preparations. Recombinant expression of each of the α_{1B}, α_2, and β forms might reveal multiple N-type channels and the functional consequence of various subunit combinations (*35*).

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20. Recombinant cDNA libraries were prepared, and overlapping $\alpha_{1B\text{-}1}$ cDNA clones were isolated from IMR32, human hippocampus, and basal ganglia cDNA libraries and characterized essentially as described (*3*, *8*).
21. We performed PCR analyses as described (*8*) using IMR32 RNA, human hippocampus RNA, and human genomic DNA with $\alpha_{1B\text{-}1}$-specific primers [nucleotides (nt) 6368 to 6391 and the complement of nt 7071 to 7095] to confirm the α_{1B} termination codon. The RNAs gave the expected 728-bp fragment ($\alpha_{1B\text{-}1}$) as well as a 541-bp fragment ($\alpha_{1B\text{-}2}$). The genomic DNA product was ~1350 bp. The DNA sequences of $\alpha_{1B\text{-}1}$ and $\alpha_{1B\text{-}2}$ diverge from each other after nt 6489. The $\alpha_{1B\text{-}1}$ subunit contains an additional 187-bp exon that alters the reading frame. After this exon, the $\alpha_{1B\text{-}1}$ and $\alpha_{1B\text{-}2}$ sequences are identical for the remaining 419 nucleotides characterized from both sequences, $\alpha_{1B\text{-}1}$ nt 6677 to 7095 and $\alpha_{1B\text{-}2}$ nt 6490 to 6908. The presence of the exon ($\alpha_{1B\text{-}1}$) results in the termination of the coding sequence at nt 7018 to 7020 (TAG); the absence of the exon ($\alpha_{1B\text{-}2}$) results in the termination of the coding sequence at nt 6712 to 6714 (TGA). Differential processing of the α_{1B} primary transcript was confirmed by characterization of the α_{1B} genomic PCR product. An ~270-bp intron was identified between $\alpha_{1B\text{-}1}$ nt 6489 and 6490. The $\alpha_{1B\text{-}1}$ and $\alpha_{1B\text{-}2}$ transcripts result from alternative selection of splice acceptor sites. $\alpha_{1B\text{-}1}$ is formed by selection of the splice acceptor at the intron-exon boundary, at nt 6490 on the exon side of the boundary; $\alpha_{1B\text{-}2}$ is formed by selection of a splice acceptor identified by an AG dinucleotide at nt 6675 and 6676 of the $\alpha_{1B\text{-}1}$ coding sequence.
22. Tissue distribution of the $\alpha_{1B\text{-}1}$ and $\alpha_{1B\text{-}2}$ transcripts was determined by PCR assays with oligonucleotide primers, nt 6447 to 6470 (Pro^{2149} to Glu^{2157}), and the complement of $\alpha_{1B\text{-}1}$-specific nt 6819 to 6843 (Leu^{2273} to Glu^{2281}). PCR products were probed with an $\alpha_{1B\text{-}1}$-specific oligonucleotide (nt 6513 to 6536; Ser^{2171} to Ala^{2179}) and an $\alpha_{1B\text{-}2}$-specific oligonucleotide (nt 6480 to 6498; Pro^{2160} to Ser^{2166}). The expected size bands were 396 bp ($\alpha_{1B\text{-}1}$) and 209 bp ($\alpha_{1B\text{-}2}$).
23. pcDNA$\alpha_{1B\text{-}1}$ was constructed in pcDNA1 (Invitrogen, San Diego, CA) with α1.179 (nt −143 to 2194), α1.177 (nt 2194 to 4160), α1.201 (nt 4160 to 5305), α1.200 (nt 5305 to 6116), and α1.230 (nt 6116 to 7176). DNA sequence analysis revealed that α1.177 has a two-nucleotide deletion (nt 3711 to 3712; Ser^{1237}) that was corrected with a PCR-amplified IMR32 Nar I–Kpn I fragment (nt 3685 to 4160; Gly^{1229} to Gly^{1387}). pHBCaCHα_{2b}(A) and pHBCaCHβ_{2b}.RBS(A), full-length α_{2b} and β_2 constructs in pcDNA1, were described previously (*8*). HEK293 cells [B. W. Stillman and Y. Gluzman, *Mol. Cell. Biol.* 5, 2051 (1985)] were grown as a monolayer culture in Dulbecco's modified Eagle's medium (Gibco) containing 5% defined-supplemented bovine calf serum (Hyclone) plus penicillin G (100 U/ml) and streptomycin sulfate (100 μg/ml). HEK293 cell transfections were mediated by calcium phosphate [F. M. Ausubel *et al.*, Eds., *Current Protocols in Molecular Biology* (Wiley, New York, 1990), pp. 9.1.1 to 9.1.7]. Cells were transfected (2×10^6 per polylysine-coated plate). Standard transfections (10-cm dish) contained 8 μg of pcDNA$\alpha_{1B\text{-}1}$, 5 μg of pHBCaCHα_{2b}(A), 2 μg of pHBCaCHβ_{2b}.RBS(A), 2 μg of pCMVβ (Clontech β-galactosidase expression plasmid), and pUC18 to maintain a constant mass of 20 μg/ml. Cells were analyzed 48 to 72 hours after transfection. Transfection efficiencies (±10%) were determined by in situ histochemical staining for β-galactosidase activity [J. R. Sanes, J. L. R. Rubenstein, J.-F. Nicolas, *EMBO J.* 5, 3133 (1986)]. Transfection efficiencies generally were >50%.
24. Properties of recombinantly expressed Ca^{2+} channels were studied by whole cell patch-clamp techniques [O. P. Hamill, A. Marty, E. Neher, B. Sakmann, F. J. Sigworth, *Pfluegers Arch.* 391, 85 (1981)]. Recordings were performed on transfected HEK293 cells 2 to 3 days after transfection. Cells were plated at 100,000 to 300,000 cells per polylysine-coated, 35-mm tissue culture dishes (Falcon, Oxnard, CA) 24 hours before recordings. Cells were perfused with 15 mM $BaCl_2$, 125 mM choline chloride, 1 mM $MgCl_2$, and 10 mM Hepes (pH = 7.3) adjusted with tetraethylammonium hydroxide (bath solution). Pipettes were filled with 135 mM CsCl, 10 mM EGTA, 10 mM Hepes, 4 mM Mg–adenosine triphosphate (pH = 7.5) adjusted with tetraethylammonium hydroxide. Sylgard (Dow-Corning, Midland, MI)-coated, fire-polished, and filled pipettes had resistances of 1 to 2 megohm before we established gigohm seals to cells. ω-CgTx (Bachem), Bay K 8644, and nifedipine (Research Biochemicals, Natick, MA) were prepared as described (*8*), dissolved in bath solution, and continuously applied by means of puffer pipettes as required for a given experiment. Recordings were performed at room temperature (22° to 25°C). Series resistance compensation (70 to 85%) was employed to minimize voltage error that resulted from pipette access resistance, typically 2 to 3.5 megohm. Current signals were filtered (−3 dB, 4-pole Bessel) at a frequency of 1/4 to 1/5 the sampling rate, which ranged from 0.5 to 3 kHz. Voltage commands were generated, and data were acquired with CLAMPEX (pClamp, Axon Instruments, Foster City, CA). All data shown are corrected for linear leak and capacitive components as described (*8*). Exponential fitting of currents was performed with CLAMPFIT (Axon).
25. Currents <30 pA were not included because of unreliable measurements. For $\alpha_{1B\text{-}1}\alpha_{2b}\beta_2$-transfected cells, currents in 43.6% of the expressing cells ranged from 30 to 200 pA, 43.6% of the cells had currents that ranged from 200 to 1000 pA, and 12.8% had currents that exceeded 1000 pA.
26. We mechanically removed cells from tissue culture plates 48 hours after transfection by spraying with phosphate-buffered saline that contained 0.1% (w/v) bovine serum albumin (BSA). The cells were collected, washed once, and resuspended in assay buffer [10 mM Hepes (pH 7.4), 140 mM NaCl, 5 mM KCl, 12 mM glucose, and BSA (1 mg/ml)]. Specific binding of ^{125}I–ω-CgTx to transfected cells was determined as described (*30*) with several modifications. Briefly, we performed the assay in 12 mm × 75 mm polypropylene tubes in 0.5 ml of assay buffer by incubating the cells with 100 pM ^{125}I–ω-CgTx (DuPont Biotechnology Systems; 2200 Ci/mmol) for 1 hour at 37°C. Subsequently, 2 ml of ice-cold wash buffer [5 mM Hepes (pH 7.4), 160 mM choline chloride, 1.5 mM $CaCl_2$, and BSA (1 mg/ml)] was added to each tube, and the mixtures were centrifuged at 2300*g* for 30 min at 4°C. The pellets were washed again and counted for radioactivity. Nonspecific binding was determined in the presence of 20 nM unlabeled ω-CgTx. The optimum cell number was determined by a titration of 1×10^5 to 2×10^6 cells per assay tube. For saturation binding studies, the binding of ^{125}I–ω-CgTx was measured as a function of increasing concentration of ^{125}I–ω-CgTx. Nonspecific binding was subtracted at each concentration. Specific binding was plotted as a function of ^{125}I–ω-CgTx concentration and analyzed by the Scatchard method.
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35. The amino acid sequence of a rat α_1 subunit, rbB-I, has been reported [S. J. Dubel *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 89, 5058 (1992)] and is 92.8% identical to that of the human $\alpha_{1B\text{-}1}$ subunit. However, attempts to express the cDNA encoding the rbB-I protein did not yield functional Ca^{2+} channels, thus supporting our conclusion that additional subunits, such as β_2 and α_{2b}, are

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Membrane Depolarization Induces p140^{trk} and NGF Responsiveness, But Not p75^{LNGFR}, in MAH Cells

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Nerve growth factor (NGF) is required for the maturation and survival of sympathetic neurons, but the mechanisms controlling expression of the NGF receptor in developing neuroblasts have not been defined. MAH cells, an immortalized sympathoadrenal progenitor cell line, did not respond to NGF and expressed neither low-affinity NGF receptor (p75) nor p140^{trk} messenger RNAs. Depolarizing concentrations of potassium chloride, but none of a variety of growth factors, induced expression of p140^{trk} but not p75 messenger RNA. A functional response to NGF was acquired by MAH cells under these conditions, suggesting that expression of p75 is not essential for this response. Depolarization also permitted a relatively high proportion of MAH cells to develop and survive as neurons in fibroblast growth factor and NGF. These data establish a relation between electrical activity and neurotrophic factor responsiveness in developing neurons, which may operate in the functioning of the mature nervous system as well.

The survival of vertebrate neurons is dependent on neurotrophic factors secreted by their postsynaptic targets. NGF, the prototypic neurotrophic factor, is required for the survival of sympathetic and some sensory neurons (*1*). The embryonic precursors to sympathetic neurons neither respond to nor require NGF (*2–4*). This raises the question of how developing sympathetic neuroblasts acquire their responsiveness to and dependence on NGF. We have studied this process with the use of MAH cells a retrovirally immortalized sympathoadrenal progenitor cell line (*5*). The identification of the product of the proto-oncogene *trk*, p140^{trk} (Trk), as a signal-transducing subunit of the NGF receptor (NGFR) (*6, 7*) has allowed us to use Trk mRNA expression to assay environmental signals that may induce NGF responsiveness in MAH cells. Here we identify membrane depolarization as one such signal.

MAH cells, like the nonimmortalized progenitors from which they derive, do not undergo neuronal differentiation in response to NGF. The protein p75, the low-affinity NGFR (*8, 9*), is not expressed by these cells (*5*). MAH cells grown in the absence of added factors also express little or no Trk mRNA (Fig. 1A, lanes 1 and 2). Thus, the failure of these precursor cells to respond to NGF correlates with their lack of expression of both types of NGFR mRNAs. We then sought to identify factors that induce expression of NGFR and NGF responsiveness. Previously, we found that basic fibroblast growth factor (bFGF) induced low levels of p75 expression and NGF responsiveness in a small subpopulation of MAH cells (*5*). However, bFGF failed to induce significant Trk expression in MAH cells, as did a number of other growth and neurotrophic factors (Fig. 1A, lanes 4 through 7, and data not shown). In addition, retinoic acid, which induces high-affinity NGF receptors and NGF dependence in chick sympathetic neuroblasts (*10*), did not induce Trk mRNA (Fig. 1A, lane 8).

In the chick, depolarization increases the survival of NGF-dependent sympathetic neurons (*4*). In MAH cells, depolarization stimulated the survival of postmitotic neurons. Depolarization of MAH cells produced by the addition of 40 mM KCl led to an induction of Trk mRNA (Fig. 1A, lane 3). A time course in 40 mM KCl revealed that Trk expression was detectable within 24 hours and reached maximal amounts within 3 days (Fig. 1C, lanes 5 through 8). Reprobing of the same blots with p75 probes revealed that, in contrast to Trk mRNA, p75 mRNA was not induced by 40 mM KCl.

MAH cells require dexamethasone (dex) for long-term survival; when dex is removed, the cells die within 4 to 5 days. In the presence of 5 μM dex, a low steady-state amount of Trk mRNA was detected (Fig. 1B, lane 2). However, even in the presence of dex an up-regulation of Trk mRNA by 40 mM KCl occurred (Fig. 1B, lane 3), indicating that the effect of depolarization is not simply to maintain the survival of Trk-expressing MAH cells. The time course of Trk induction by 40 mM KCl was similar in the presence of dex (Fig. 1D, lanes 6 through 9) as in its absence, although higher steady-state amounts of Trk mRNA were produced in the presence of dex (compare Fig. 1D, lane 8, with Fig. 1C, lane 7). As was the case in the absence of dex, no induction of p75 mRNA was detected in 40 mM KCl plus dex.

The effect of 40 mM KCl is likely to be produced by membrane depolarization because no induction of Trk mRNA was observed in 40 mM NaCl (Fig. 1, A and B, lanes 9). Moreover, veratridine, an Na^+ channel agonist that leads to membrane depolarization, also caused an increase in the amount of Trk mRNA concentrations (data not shown). In PC12 cells, the induction of immediate-early gene expression by membrane depolarization requires the opening of voltage-gated Ca^{2+} channels and depends on extracellular Ca^{2+} (*11*). Removal of extracellular Ca^{2+} or addition of dihydropyridine antagonists of voltage-gated Ca^{2+} channels resulted in the death of MAH cells within 24 hours, precluding our ability to determine a requirement for Ca^{2+} influx in Trk induction. However, at suboptimal concentrations of KCl (20 mM) (Fig. 1E, lane 4), the Ca^{2+} channel agonist Bay K 8644 potentiated the induction of Trk mRNA (Fig. 1F, lanes 3 and 4), which suggests that Ca^{2+} influx through voltage-gated L-type Ca^{2+} channels is indeed involved in the induction of Trk mRNA by membrane depolarization.

We then sought to determine whether depolarization induces a functional response to NGF. We used two assays of NGF responsiveness: neurite outgrowth and cell number. Cell number reflects both the survival- and proliferation-promoting (*12*) effects of NGF, although for technical reasons it is difficult to determine the relative contributions of these two processes in this system. NGF responses were assayed after 5 days, by which time most MAH cells had died in control medium (Table 1). Those few cells that survived showed little process outgrowth (Fig. 2A). Similar results were obtained in NGF alone (Fig. 2B and Table 1), indicating that MAH cells do not respond to this factor. Cell number was significantly increased by depolarizing concentrations of KCl (Table 1), although little neurite outgrowth was observed (Fig. 2C). In NGF plus 40 mM KCl, cell number was even higher (Table 1) and the cells bore long neurites (Fig. 2D). These neurite-bearing cells, however, lacked the cell soma hypertrophy characteristic of mature neu-

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Structure and Functional Expression of α_1, α_2, and β Subunits of a Novel Human Neuronal Calcium Channel Subtype

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Summary

The primary structures of human neuronal α_1, α_2, and β subunits of a voltage-dependent Ca^{2+} channel were deduced by characterizing cDNAs. The α_1 subunit (α_{1D}) directs the recombinant expression of a dihydropyridine-sensitive L-type Ca^{2+} channel when coexpressed with the β (β_2) and the α_2 (α_{2b}) subunits in Xenopus oocytes. The recombinant channel is also reversibly blocked by 10–15 μM ω-conotoxin. Expression of the α_{1D} subunit alone, or coexpression with the α_{2b} subunit, did not elicit functional Ca^{2+} channel activity. Thus, the β_2 subunit appears to serve an obligatory function, whereas the α_{2b} subunit appears to play an accessory role that potentiates expression of the channel. The primary transcripts encoding the α_{1D}, α_2, and β subunits are differentially processed. At least two forms of neuronal α_{1D} were identified. Different forms of α_2 and β transcripts were also identified in CNS, skeletal muscle, and aorta tissues.

Introduction

The primary pathway by which Ca^{2+} enters excitable cells is through voltage-dependent Ca^{2+} channels present in cellular membranes (Bean, 1989). Multiple subtypes of these channels have been identified (Hess, 1990), the best characterized of which is the rabbit skeletal muscle dihydropyridine (DHP)-sensitive Ca^{2+} channel, consisting of four tightly coupled subunits, α_1, α_2, β, and γ (Campbell et al., 1988). Each of these subunits has been characterized by cDNA cloning (Tanabe et al., 1987; Ellis et al., 1988; Ruth et al., 1989; Jay et al., 1990). Recent evidence suggests that different α_1 subunits are encoded by a gene family comprising at least five distinct genes, some of which are expressed in several tissues (Ellis et al., 1988; Mikami et al., 1989; Perez-Reyes et al., 1990; Snutch et al., 1990). The gene encoding the α_1 subunit expressed in rabbit skeletal muscle directs the recombinant expression of a functional DHP-sensitive Ca^{2+} channel in cultured myotubes of *mdg* mice and in mouse L cells (Tanabe et al., 1988; Perez-Reyes et al., 1989). A second gene, encoding α_1 subunits expressed in rabbit cardiac and lung tissues, directs the synthesis of DHP-sensitive Ca^{2+} channels in Xenopus oocytes (Mikami et al., 1989; Biel et al., 1990). In contrast, a third α_1 subunit gene, expressed in rabbit brain, directs the synthesis of Ca^{2+} channels that are insensitive to both DHPs and ω-conotoxin GVIA (ω-CgTx) when coexpressed with the rabbit skeletal muscle α_2 and β subunits in Xenopus oocytes (Mori et al., 1991). These expression studies in oocytes demonstrated that the α_1 subunit forms the pore through which Ca^{2+} enters the cell. The functional expression of α_1 subunits encoded by the two remaining genes has not yet been reported.

The entry of Ca^{2+} through voltage-dependent Ca^{2+} channels in neurons controls diverse functions, such as neurotransmitter release, excitability, and differentiation (Tsien et al., 1988). On the basis of biophysical and pharmacological characterizations, four subtypes of neuronal voltage-dependent Ca^{2+} channels have been proposed (Llinàs et al., 1989; Swandulla et al., 1991). Although specific neuronal functions have been ascribed to different Ca^{2+} channel subtypes, the analysis has been difficult due to the coexistence of multiple subtypes in individual cells (Miller, 1987; Bean, 1989; Hess, 1990; Swandulla et al., 1991). One important step in defining subtype–function relationships is the cloning and expression of each neuronal subtype as a pure population. We report the complete amino acid sequence and functional expression of three subunits of a human neuronal L-type voltage-dependent Ca^{2+} channel: an α_1 subunit (designated α_{1D}), an α_2 subunit (designated α_{2b}), and a β subunit (designated β_2). A description of the nomenclature used to designate the different Ca^{2+} channel subunits is provided in the Experimental Procedures. We also report tissue-specific processing of the α_2 and β transcripts.

Results

Cloning and Characterization of Three Human Neuronal Voltage-Dependent Ca^{2+} Channel Subunits

We previously reported the isolation of cDNAs that encode the α_1, α_2, β, and γ subunits of the rabbit skeletal muscle DHP-sensitive, L-type Ca^{2+} channel (Ellis et al., 1988; Jay et al., 1990). These subunit cDNAs were used as probes to isolate related human neuronal cDNAs as described in the Experimental Procedures. The primary structures of the human neuronal α_{1D}, α_{2b}, and β_2 subunits (Figure 1; see Figure 3 and Figure 4) were deduced from these cDNA sequences.

α_{1D} Subunit

The primary structure of the human α_{1D} subunit (Figure 1) comprises 2161 amino acids, yielding a calculated molecular weight of 245,163. The α_{1D} sequence is most similar (96.3% deduced amino acid sequence identity) to the previously reported 188 amino acid

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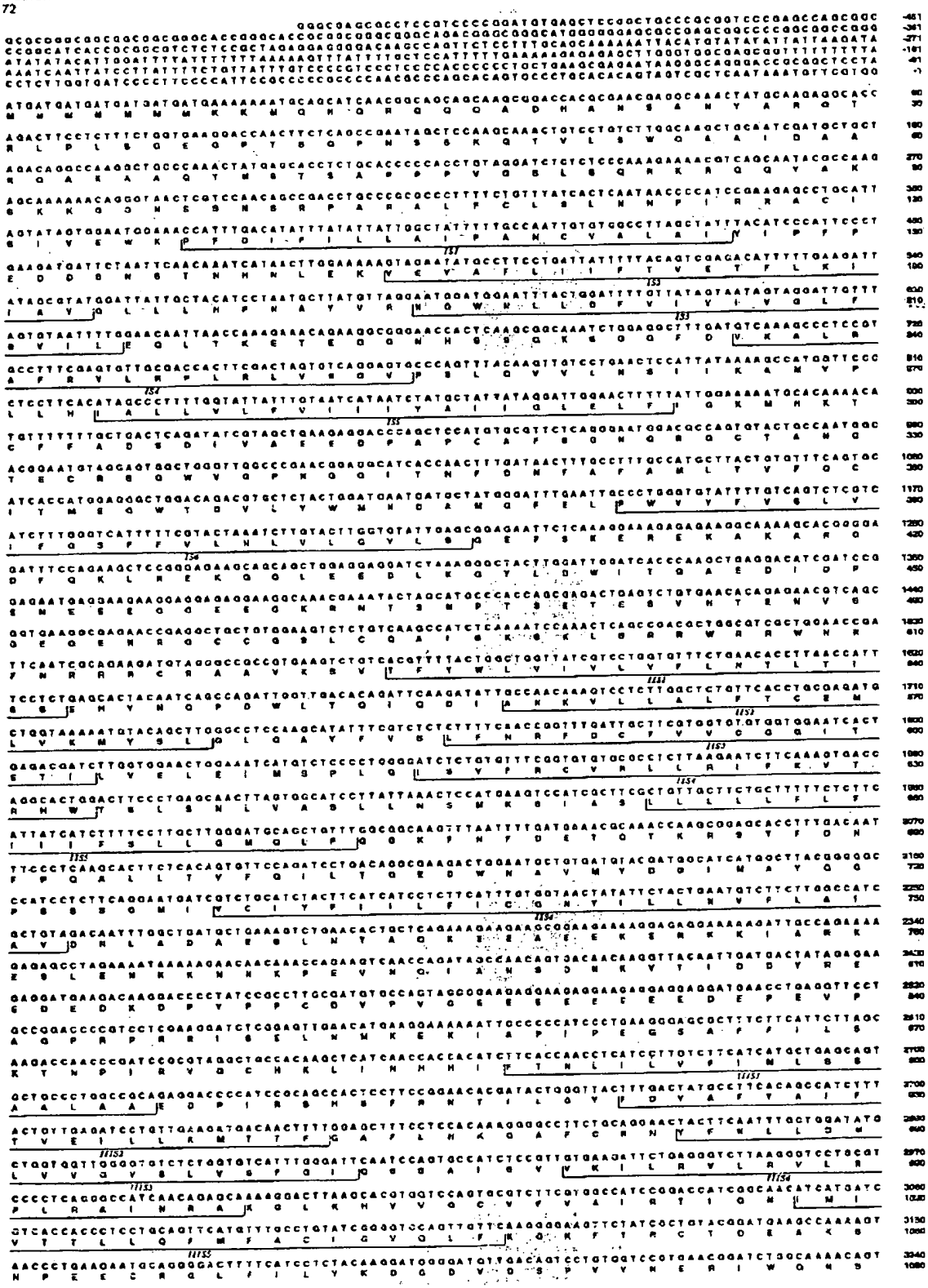
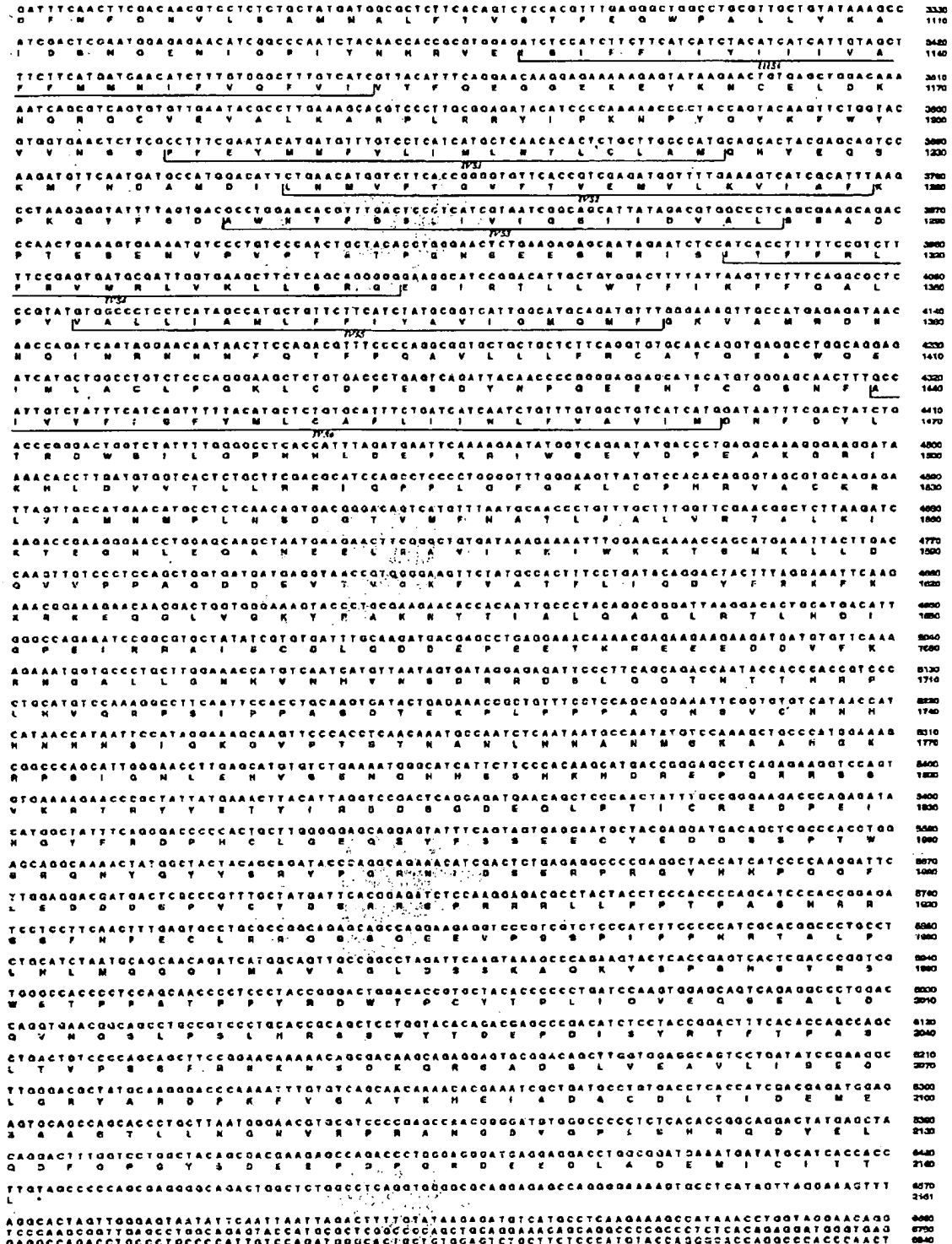


Figure 1. α_{1D} Nucleotide and Deduced Amino Acid Sequences

The 5′ untranslated sequence is negatively numbered. Positive numbering begins at the first nucleotide of the proposed initiating codon. The number of the nucleotide and amino acid residue is given at the end of each line. The proposed transmembrane segments S1–S6 in each of the repeats I–IV are shown (brackets).



partial rat brain class D cDNA (Snutch et al., 1990). The translation initiation site was assigned to the first methionine codon that appears downstream of an in-frame nonsense codon. Interestingly, 7 methionine codons appear at the beginning of the putative coding sequence, followed by 2 lysine codons and an eighth methionine codon; none of these methionine codons are contained within the consensus sequence for eucaryotic initiation codons (Kozak, 1987). This series of methionine codons was confirmed by direct sequence analysis of cloned polymerase chain reaction (PCR) products derived from reactions performed on human neuroblastoma IMR32 cell cytoplasmic RNA, as described in the Experimental Procedures.

The predicted structure of the α_{1D} subunit consists of four repeating domains, each domain comprised of five hydrophobic segments (S1, S2, S3, S5, and S6) and one positively charged segment (S4), suggesting the same transmembrane topology as described previously for Ca^{2+} channel α_1 subunits and Na^+ channels (Numa and Noda, 1986; Tanabe et al., 1987; Mikami et al., 1989; Biel et al., 1990; Koch et al., 1990; Mori et al., 1991). Based on this proposed topology, the α_{1D} subunit has 3 of 12 potential N-glycosylation sites (Bause, 1983) assigned to the extracellular side and nine of ten potential cAMP-dependent phosphorylation sites (Glass et al., 1986) and 22 of 26 potential protein kinase C phosphorylation sites (Woodgett et al., 1986) assigned to the cytoplasmic side of the cellular membrane (Figure 2).

The α_{1D} cDNA clone α1.136 was found to encode an incompletely processed transcript containing two exons encoding the IS6 transmembrane domain, designated α_{1D} exon A and α_{1D} exon B. The deduced amino acid sequences are MNDAMGFELPWVYFVSL-VIFGSFFVLNLVLGVLSG and VNDAIGWEWPWVYFV-SLIILGSFFVLNLVLGVLSG, respectively, which share 83% identity. Exon A was present in clone α1.144, which was used for the construction of the full-length α_{1D} cDNA used in the present study (Figure 1).

The deduced amino acid sequences of two different α_1 subunits, the rabbit cardiac (Mikami et al., 1989) and the rabbit brain BI-2 (Mori et al. 1991), previously expressed in Xenopus oocytes, are shown aligned with the human α_{1D} sequence (Figure 2). The amino acid sequence identity of α_{1D} to these sequences is significant: 70.3% and 40.5% for the cardiac and BI-2 sequences, respectively. The sequence identity is well conserved through the four repeating domains, 79.7% and 50.5% for the α_{1D}–cardiac and the α_{1D}–BI-2 pairs, respectively. Most noteworthy is the divergence of the α_{1D} and cardiac sequences compared with the BI-2 sequence through the putative DHP-binding region (Regulla et al., 1991). In this region, the α_{1D} and cardiac DHP-sensitive forms differ by a single amino acid (Ser-1490) as does the rabbit skeletal muscle sequence (Ala-1404), whereas the BI-2 DHP-insensitive form has 18 amino acid substitutions in this region (Figure 2). This evidence, together with the results of the expression studies reported here (see below), supports the proposed identity of the DHP-binding region.

α_{2b} Subunit

The primary structure of the human brain α_{2b} subunit (Figure 3) consists of 1091 amino acids, yielding a calculated molecular weight of 123,182. The amino acid sequence homology is 97.1% identical to the rabbit skeletal muscle α_{2a} subunit sequence (Figure 3) and has essentially an identical predicted topography and secondary structure (Ellis et al., 1988; Jay et al., 1991), with the exceptions of a 19 amino acid deletion in the human sequence compared with the rabbit sequence (α_{2a} residues Pro-507 to Gln-525) and a 7 amino acid insertion in the human sequence compared with the rabbit sequence (α_{2b} residues Lys-602 to Asp-608). The 16 potential glycosylation sites that were identified in the rabbit skeletal muscle α_{2a} subunit (Jay et al., 1991) also are conserved in the human α_{2b} sequence. Previous studies suggest that posttranslational processing of the rabbit skeletal α_{2a} subunit results in a heterogeneous population of δ peptides, all of which begin at Ala-935 (Jay et al., 1991). The human brain α_{2b} sequence has two conservative amino acid substitutions at this proposed cleavage site, Val-923 and Glu-924 replacing Ala-935 and Asp-936, respectively (Figure 3).

β_2 and β_3 Subunits

The primary structure of the human brain β_2 subunit (Figure 4) comprises 478 amino acids and has a calculated molecular weight of 52,934. The amino acid sequence homology is 96.9% identical to the rabbit skeletal muscle β_1 subunit sequence (Figure 4). The β_2 subunit has essentially an identical topography and secondary structure as predicted for the rabbit skeletal muscle β_1 subunit (Ruth et al., 1989) except that the deduced human β_2 sequence has a deletion of 45 amino acids (Ala-217 to Lys-261). The lack of this region in β_2 removes the second α helical domain proposed for the rabbit skeletal muscle β_1 subunit (Ruth et al., 1989). Thirteen of sixteen potential phosphorylation sites identified in the rabbit skeletal muscle β_1 subunit (Ruth et al., 1989) are conserved in the human β_2 sequence (Figure 4). Two sites are changed due to amino acid substitutions (β_1 Ser-179 and Ser-182), and the third is removed by the 45 amino acid deletion (β_1 Ser-238).

Another form of β, designated β_3, which has the same deduced 45 amino acid deletion, was identified in the hippocampus cDNA library. Clone λβ4 encodes the β_3 cDNA and diverges from β_2 after nucleotide 1332. The β_3 cDNA extends another 429 nucleotides with no translation stop codon identified (data not shown). A GT splice donor is not present at the point of divergence between the β_2 and β_3 sequences. A complete characterization of β_3 is in progress.

Tissue-Specific Processing of the α_2 and β Transcripts and Distribution of α_{1D}, α_2, and β mRNAs

PCR analysis and hybridization with oligonucleotides derived from α_{2a}– or α_{2b}–specific regions (the 19 amino acid region or the 7 amino acid region, respectively; Figure 3) demonstrated that the human skeletal

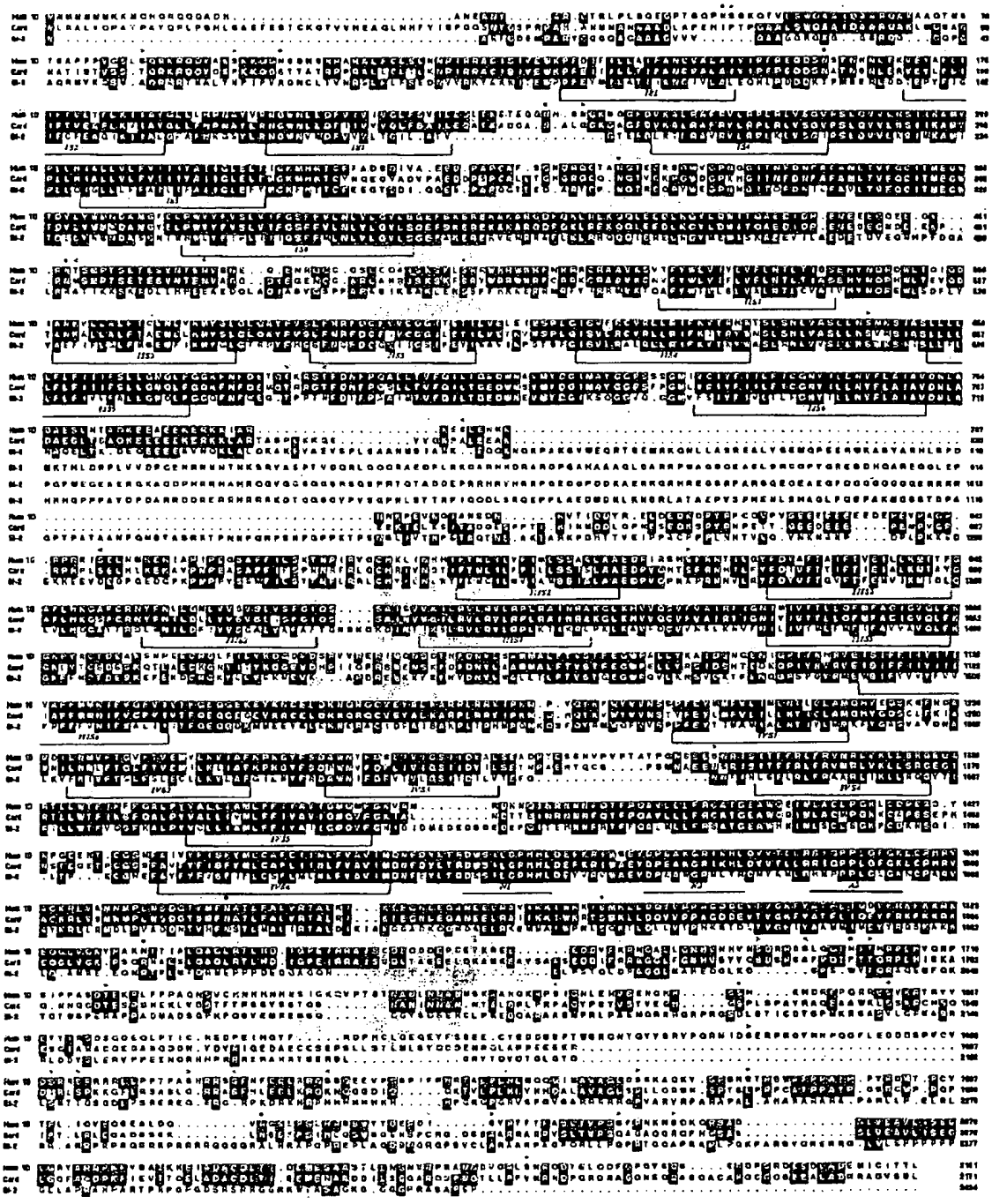


Figure 2. Alignment of Deduced Amino Acid Sequences of α_1 Subunits

The amino acid sequences of the human neuronal Ca^{2+} channel α_{1D} (Hum 1D), the rabbit cardiac α_1 (Card; Mikami et al., 1989), and the rabbit brain α_1 (BI-2; Mori et al., 1991) are shown in single-letter code. The numbering begins with the proposed initiating methionine. The number of the amino acid residue at the end of each line is given. Identical residues at one position in two of the three sequences are shown as white letters on black background. The putative transmembrane segments S1–S6 in each of the repeats I–IV are shown (brackets). The putative DHP-binding regions (N1, N2, A2; Regulla et al., 1991) are shown (underlined). Potential N-glycosylation (o), cAMP-dependent phosphorylation (<), and protein kinase C phosphorylation (>) sites are shown. Potential targets of either kinase are labeled (+).

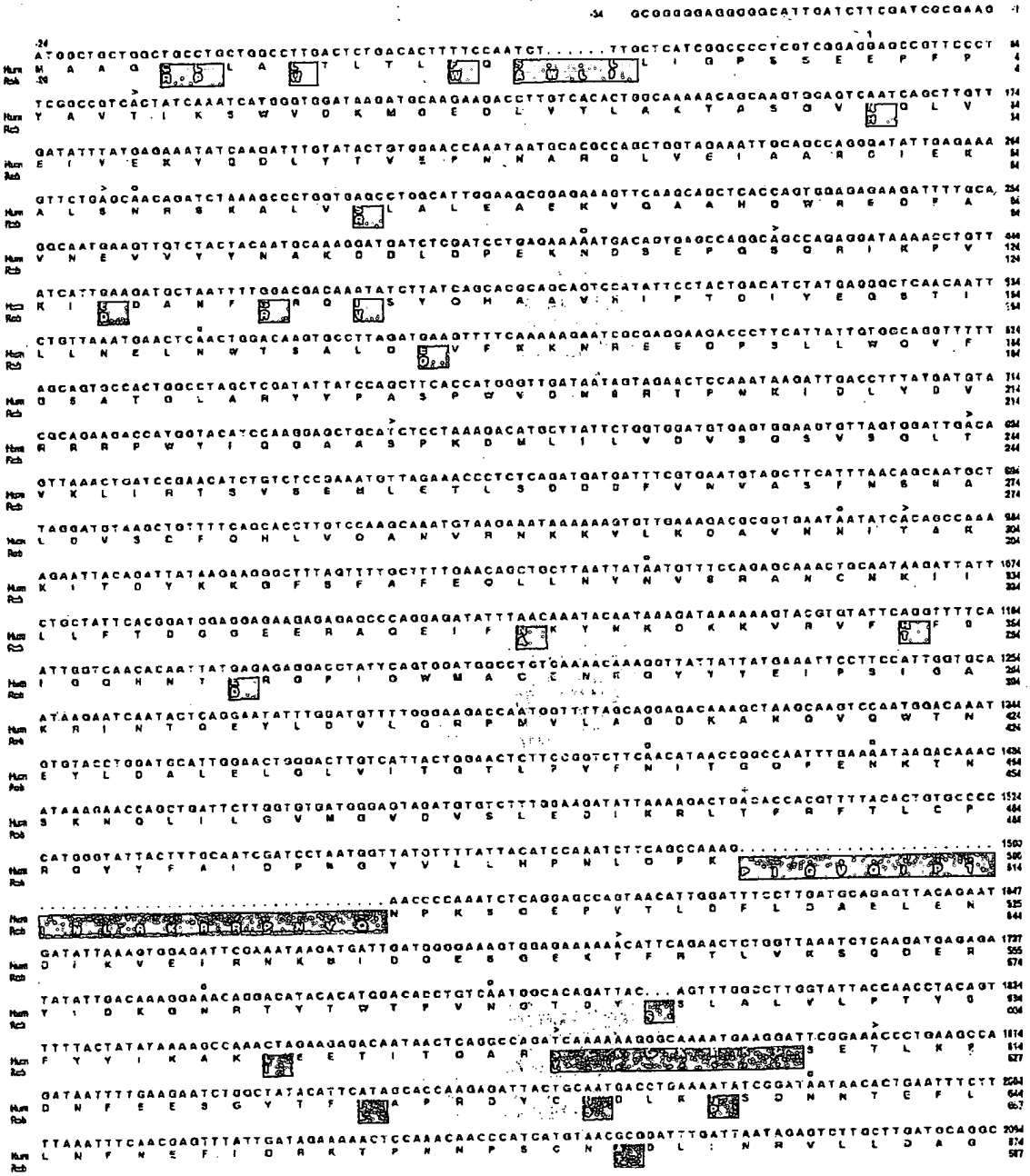
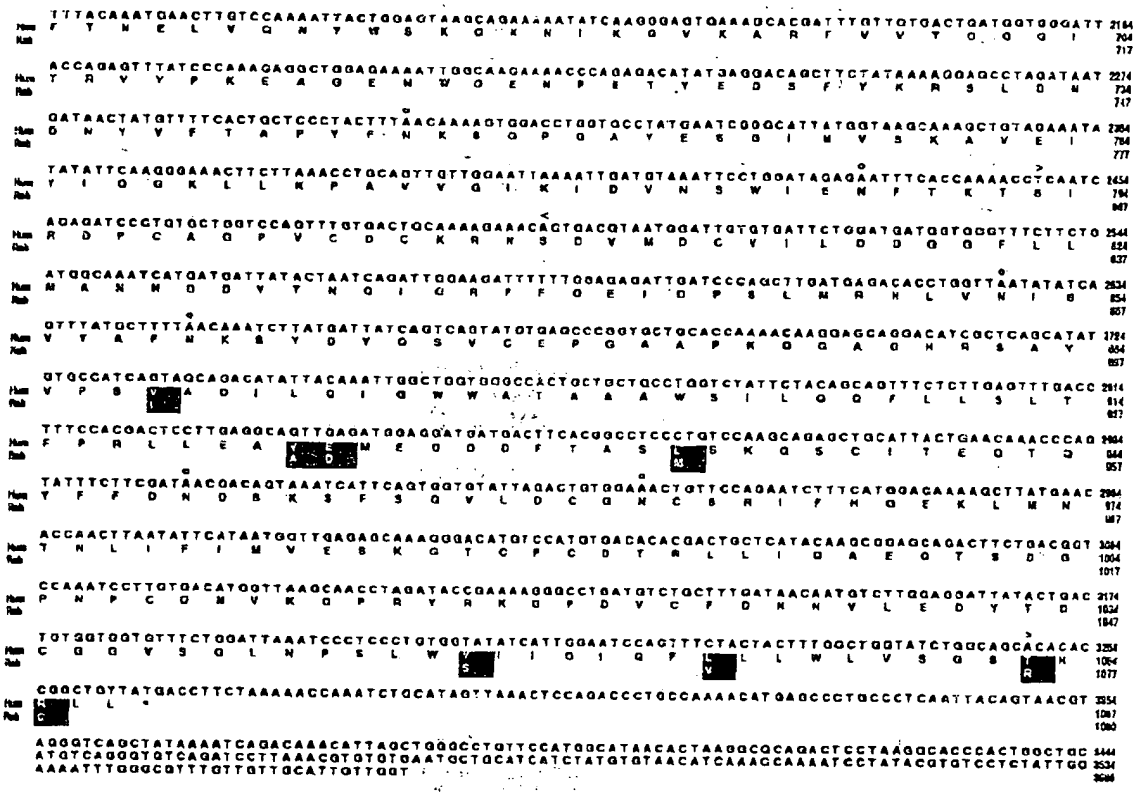


Figure 3. Determined cDNA Sequence of α_{2b} and Alignment of the Deduced Amino Acid Sequence with the Deduced Rabbit Skeletal Muscle α_{2a} Sequence

For the rabbit α_{2a} sequence (Rab; Ellis et al., 1988), only the amino acid differences compared with the human α_{2b} sequence (Hum) are shown. The 5′ untranslated sequence is negatively numbered. Positive numbering begins at the first nucleotide of the proposed initiating codon. The number of the nucleotide and amino acid residue is given at the end of each line. Negative numbers for amino acids designate residues contained in the proposed signal sequence (Ellis et al., 1988) beginning with the initiating methionine (−24 and −26). The positive numbering begins at the NH_2−terminal residue (glutamic acid) of the mature protein. Amino acid sequence differences and insertions/deletions are identified by the black boxes. Potential N-glycosylation (o), cAMP-dependent phosphorylation (<), and protein kinase C phosphorylation (>) sites are shown. The potential target of either kinase is labeled (+).



muscle α_2 transcript is processed in a manner similar to the rabbit skeletal muscle transcript (540 bp bands; Figure 5A). Furthermore, the α_2 transcripts expressed in IMR32 cells and human CNS tissues (501 bp bands; Figure 5B) and the α_2 transcript expressed in aorta tissue (490 bp band; Figure 5B) are processed differentially to yield at least two additional α_2 transcript species, α_{2b} (Figure 3) and α_{2c}, respectively.

PCR analysis of β-specific RNAs showed that the β primary transcript is also processed in a tissue-specific manner. Analysis of human skeletal muscle RNA detected the 135 nucleotides absent in β_2 (Figure 4) and, thus, confirmed the presence of a distinct skeletal muscle β_1 transcript (Figure 5C). In addition to the β_1 form expressed in skeletal muscle and the β_2 and β_3 forms expressed in the CNS, another form, designated β_4, was detected in aorta tissue having a 156 nucleotide deletion relative to the skeletal muscle β_1 transcript (Figure 5C).

To confirm the tissue-specific processing of the β subunit primary transcript, β-specific PCR products of human skeletal muscle and aorta were cloned, and the DNA sequence was determined. The deduced human skeletal muscle amino acid sequence is 92% identical to the rabbit skeletal muscle sequence from position Gly-210 to Lys-261 (Figure 4). The β_2 sequence has a proposed alternative exon (Ala-210 to Ser-216) that probably corresponds to either the human skeletal muscle sequence Gly-210 to Leu-216 (GNEMTNL) or Arg-255 to Lys-261 (RIPFFKK). The deduced aorta sequence lacks the region between residues Ser-209 and Thr-217 (Figure 4).

PCR analysis performed on RNAs isolated from several human primary tissues and IMR32 cells identified an α_{1D} transcript in IMR32 cells and each of the human CNS tissues, but not in human skeletal muscle (Figure 5D). An α_2 transcript was detected in all RNAs analyzed (Figures 5A and 5B), as was a β transcript (Figure 5C).

Functional Expression in Xenopus Oocytes

The expression of the human neuronal α_{1D}, α_{2b}, and β_2 subunits was studied in Xenopus oocytes. mRNAs encoding each subunit were synthesized in vitro and were injected into oocytes either alone or in various combinations. The oocytes then were examined for inward Ba^{2+} currents (I_{Ba}) mediated by voltage-dependent Ca^{2+} channels.

Oocytes coinjected with the α_{1D}, α_{2b}, and β_2 mRNAs expressed sustained I_{Ba} upon depolarization (162 ± 121 nA, n = 46) that typically showed little inactivation during test pulses ranging from 140–700 ms (Figure 6A). A series of voltage steps revealed currents that appeared at approximately −30 mV and peaked at approximately 0 mV (Figure 6B). Application of the DHP

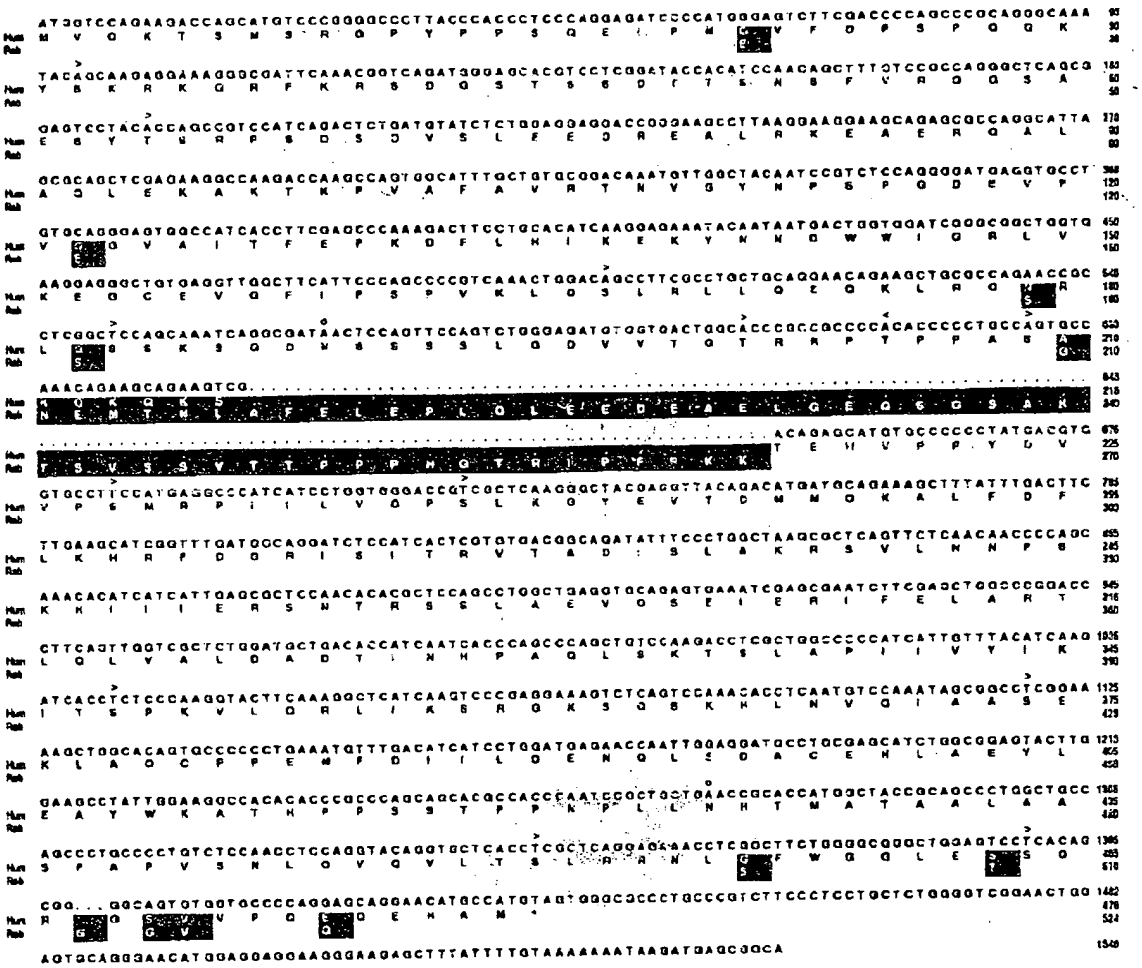


Figure 4. Determined cDNA Sequence of β_2 and Alignment of the Deduced Amino Acid Sequence with the Deduced Rabbit Skeletal Muscle β_1 Sequence

For the rabbit β_1 sequence (Rab; Ruth et al., 1989), only the amino acid differences compared with the human β_2 sequence (Hum) are shown. See legend of Figure 3 for description of symbols and numbering.

Ca^{2+} channel agonist Bay K 8644 increased the magnitude of the $\alpha_{1D}\alpha_{2b}\beta_2$–mediated currents, prolonged the "tail" currents present upon repolarization of the cell, and induced a hyperpolarizing shift in current activation (Figures 6A and 6B). Application of the DHP Ca^{2+} channel antagonist nifedipine blocked a substantial fraction of the I_{Ba} in oocytes coinjected with α_{1D}, α_{2b}, and β_2 (91% ± 6%, n = 7; Figure 6C). Much of the I_{Ba} recovered when the holding potential was shifted from −50 mV to −90 mV (data not shown), consistent with the voltage-dependent block by nifedipine (Bean, 1984; Sanguinetti and Kass, 1984). A residual inactivating component of I_{Ba} typically remained after nifedipine application. Consistent with previous studies on neuronal L-type Ca^{2+} channels (Fox et al., 1987), the $\alpha_{1D}\alpha_{2b}\beta_2$–mediated I_{Ba} was blocked completely by 50 μM Cd^{2+}, but only approximately 15% by 100 μM Ni^{2+}.

The $\alpha_{1D}\alpha_{2b}\beta_2$–mediated I_{Ba} was blocked weakly (54% ± 29%, n = 7) and reversibly by relatively high concentrations (10–15 μM) of ω-CgTx (Figure 6D). Bay K 8644 was first applied to the cell in order to determine the effect of ω-CgTx on the DHP-sensitive current component that was distinguished by the prolonged tail currents. Both the test currents and the accompanying tail currents were blocked progressively within 1–3 min after application of ω-CgTx, but both recovered partially as the ω-CgTx was flushed from the bath.

The contribution of the α_{2b} and β_2 subunits to the $\alpha_{1D}\alpha_{2b}\beta_2$–mediated current was assayed by expression of the α_{1D} subunit alone or in combination with either the β_2 subunit or the α_{2b} subunit. Oocytes injected with only the α_{1D} mRNA produced no discernable I_{Ba} upon depolarization (n = 10). Oocytes coinjected with the α_{1D} and β_2 mRNAs expressed I_{Ba} (108 ± 39 nA, n = 4) that resembled the $\alpha_{1D}\alpha_{2b}\beta_2$–mediated currents,

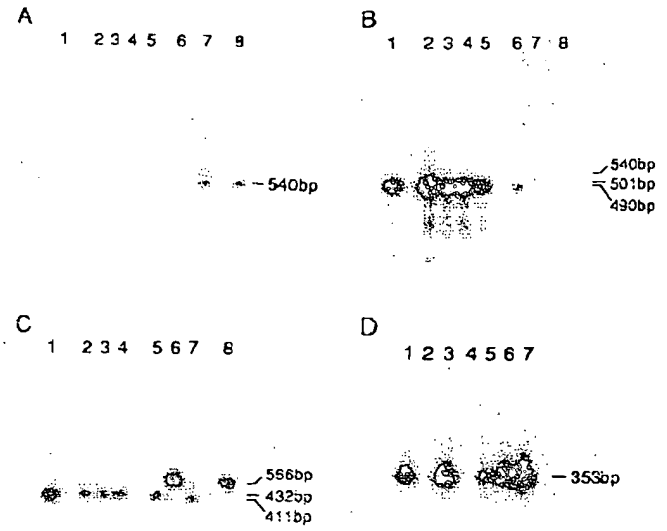


Figure 5. Autoradiographs of PCR Products Showing Distribution of Voltage-Dependent Ca^{2+} Channel Subunit Transcripts and Alternative Splicing of the α_2 and β Transcripts

IMR32 cytoplasmic RNA and human primary tissue poly(A)$^+$ RNAs were used as templates to synthesize cDNA prior to PCR analysis.

(A and B) PCR products of pHBCaCHα_2+, a human brain α_{2b} cDNA clone (lane 1), IMR32 cells (lane 2), hippocampus and basal ganglia (lane 3), habenula (lane 4), thalamus (lane 5), aorta (lane 6), skeletal muscle (lane 7), and pα2.15Δ5′, a rabbit skeletal muscle α_{2a} clone (lane 8), were hybridized with (A) an α_{2a} oligonucleotide (nucleotides 1597–1619 corresponding to Pro-507 to Thr-514; Ellis et al., 1988) or (B) an α_{2b} oligonucleotide (nucleotides 1876–1896 corresponding to Lys-602 to Asp-608). The PCR reactions were primed with human α_{2b} oligonucleotides, nucleotides 1455–1479, and the complement of nucleotides 1931–1955. An approximately equal mass of DNA was present in each lane. The sizes of the 490 bp, 501 bp, and 540 bp α_2–specific PCR products derived from human aorta, IMR32 cell and CNS tissues, and skeletal muscle RNAs, respectively, were further confirmed by electrophoresis through a 1% agarose/2% NuSieve composite gel. The weaker hybridization of the human α_{2b}–derived oligonucleotide with the 490 bp aorta and 540 bp skeletal muscle PCR products further supports their difference from the α_{2b} transcript. Each band observed also hybridized with an α_2 tissue nonspecific probe, nucleotides 1601–1626 (data not shown).

(C) PCR products of pHBCaCHβ1, a human brain β_2 cDNA clone (lane 1), IMR32 cells (lane 2), hippocampus and basal ganglia (lane 3), habenula (lane 4), thalamus (lane 5), skeletal muscle (lane 6), aorta (lane 7), and pRSKmCaCHβ2, a rabbit skeletal muscle β_1 cDNA clone (lane 8), were hybridized with a β_2 oligonucleotide, nucleotides 753–784. The PCR products were primed with β_2 oligonucleotides, nucleotides 541–560, and the complement of nucleotides 953–972.

(D) PCR products of pVDCCIII(A), an α_{1D} cDNA clone (lane 1), human genomic DNA (lane 2), IMR32 cells (lane 3), skeletal muscle (lane 4), hippocampus and basal ganglia (lane 5), habenula (lane 6), and thalamus (lane 7), were hybridized with an α_{1D} oligonucleotide, nucleotides 164–187. The PCR products were primed with α_{1D} oligonucleotides, nucleotides −39 to −18, and the complement of nucleotides 201–314.

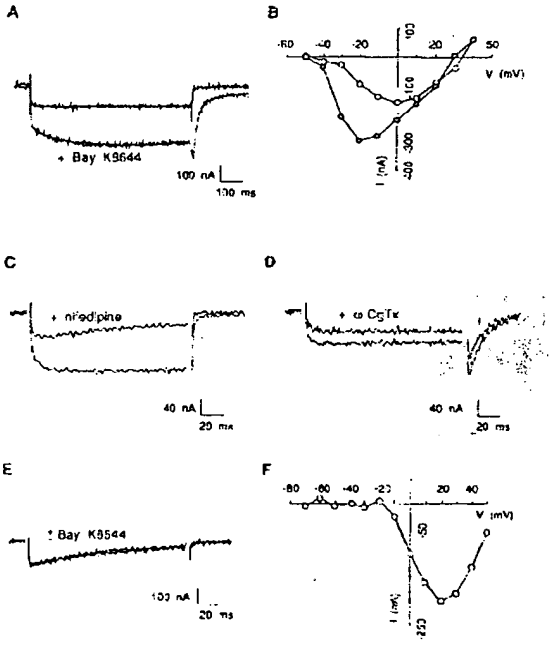


Figure 6. Functional Expression of α_{1D}, α_{2b}, and β_2 in Xenopus Oocytes

(A) I_{Ba} recorded before and after application of Bay K 8644 (1 μM) in an oocyte injected with α_{1D}, α_{2b}, and β_2 mRNAs. Test pulse, −10 mV; holding potential, −50 mV.

(B) Peak current–voltage relations before (open circles) and after (closed circles) application of Bay K 8644 for the α_{1D}, α_{2b}, and β_2 mRNA-injected cell of (A). Holding potential, −50 mV.

(C) Currents before and after (+) application of nifedipine (5 μM) in an oocyte injected with α_{1D}, α_{2b}, and β_2 mRNAs. Current traces are signal averages of three traces before and three traces after application of nifedipine. Test pulse, 0 mV; holding potential, −50 mV.

(D) Currents in the absence and presence (+) of ω-CgTx (10 μM) in an oocyte injected with α_{1D}, α_{2b}, and β_2 mRNAs. Bay K 8644 (1 μM) was present throughout. Current traces are signal averages of three traces before and three traces after application of ω-CgTx for approximately 1.5 min. Test pulse, 0 mV; holding potential, −50 mV.

(E) Currents before and after application of Bay K 8644 (1 μM) in an oocyte injected with α_{2b} and β_2 mRNAs. Superimposed current traces are signal averages of four traces before and four traces after application of Bay K 8644. Test pulse, 20 mV; holding potential, −90 mV.

(F) Peak current–voltage relation for the α_{2b} and β_2 mRNA-injected cell of (E). Holding potential, −90 mV.

although the magnitude of the current was, on average, smaller. Two of four oocytes injected with $\alpha_{1D}\beta_2$ responded to Bay K 8644 application similarly to the $\alpha_{1D}\alpha_{2b}\beta_2$–mediated currents, whereas the remaining two showed no response. Three of five oocytes coinjected with the α_{1D} and α_{2b} mRNAs displayed very small currents (15–30 nA) and were unresponsive to Bay K 8644.

To ensure that the currents observed in the $\alpha_{1D}\alpha_{2b}\beta_2$–injected oocytes were mediated by the α_{1D} subunit, expression of the β_2 or α_{2b} subunits alone or both together was assayed. Oocytes injected with the α_{2b} mRNA displayed no detectable I_{Ba} ($n = 5$). Surprisingly, oocytes injected with β_2 mRNA displayed I_{Ba} upon depolarization (54 ± 23 nA, $n = 5$), and $\alpha_{2b}\beta_2$–injected oocytes displayed I_{Ba} (Figure 6E) approximately 50% larger than the I_{Ba} of β_2–injected oocytes (81 ± 60 nA, $n = 21$). Oocytes injected with the β_2 mRNA or the α_{2b} and β_2 mRNAs together displayed I_{Ba} that typically was observed first at −30 mV and that peaked at 10–20 mV (Figure 6F). Macroscopically, the β_2– and $\alpha_{2b}\beta_2$–induced currents were indistinguishable.

In contrast to the $\alpha_{1D}\alpha_{2b}\beta_2$–mediated currents, the β_2 and $\alpha_{2b}\beta_2$ currents showed both a significant inactivation during the test pulse and a strong sensitivity to the holding potential. The I_{Ba} observed in oocytes coinjected with α_{2b} and β_2 mRNAs usually inactivated markedly during a 140 ms pulse (Figure 6E). Changing the holding potential of oocytes coinjected with the α_{2b} and β_2 mRNAs from −90 mV to −50 mV reduced the I_{Ba} 81% ± 15% ($n = 11$). In contrast, I_{Ba} measured in oocytes coinjected with the $\alpha_{1D}\alpha_{2b}\beta_2$ mRNAs was reduced 24% ± 16% ($n = 11$) when the holding potential was changed from −90 mV to −50 mV.

The $\alpha_{2b}\beta_2$–mediated I_{Ba} was also pharmacologically distinct from the $\alpha_{1D}\alpha_{2b}\beta_2$–mediated current. Oocytes coinjected with α_{2b} and β_2 mRNAs displayed I_{Ba} that was insensitive to Bay K 8644 ($n = 11$; Figure 6E). Nifedipine sensitivity was difficult to measure because of the holding potential sensitivity of both nifedipine and the $\alpha_{2b}\beta_2$–mediated I_{Ba}. Nevertheless, two oocytes coinjected with the α_{2b} and β_2 mRNAs displayed measurable I_{Ba} (25–45 nA) when depolarized from a holding potential of −50 mV, and these currents were insensitive to nifedipine (5–10 μM) application. The $\alpha_{2b}\beta_2$–mediated I_{Ba} showed a sensitivity to heavy metals similar to the $\alpha_{1D}\alpha_{2b}\beta_2$–mediated current.

Discussion

Distinct Neuronal Ca^{2+} Channel Subunits Comprise a Novel DHP-Sensitive Subtype

Our results demonstrate that the α_{1D} subunit mediates DHP-sensitive, high voltage–activated, long-lasting Ca^{2+} channel activity (Figure 6A). Significant functional expression in oocytes of the α_{1D} subunit is dependent on the coexpression of the β_2 subunit and is enhanced by coexpression with the α_{2b} subunit. The biophysical properties of activation and inactivation kinetics and voltage sensitivity of the channel formed by the α_{1D}, α_{2b}, and β_2 subunits are generally consistent with previous characterizations of neuronal L-type Ca^{2+} channels (Bean, 1989; Hess, 1990; Swandulla et al., 1991).

Immunoprecipitation of a neuronal DHP receptor previously has revealed the presence of an α_1, α_2, and β subunit complex (Ahlijanian et al., 1990). As an initial step toward a detailed characterization of the multiple subtypes of neuronal voltage-dependent Ca^{2+} channels, we cloned and expressed the human neuronal α_{1D}, α_{2b}, and β_2 subunits. Characterization of these clones revealed that both the α_{1D} and β transcripts expressed in neuronal tissue are differentially processed. Alternatively spliced α_{1D} transcripts involve at least four regions: the IS6 region reported here, the cytoplasmic loop between IS6 and IIS1 (Hui et al., 1991; data not shown), the IVS3 region, and the extracellular loop between the IVS3 and IVS4 regions (Perez-Reyes et al., 1990). In addition, a recent report described a possible form of α_{1D} with a truncated carboxyl terminus, although the functional significance of this form is unknown (Hui et al., 1991). Minimally, three forms of the α_2 subunit exist (Figures 5A and 5B): α_{2a}, expressed in skeletal muscle (Ellis et al., 1988); α_{2b}, expressed in neuronal tissues; and α_{2c}, expressed in aorta. At least four forms of the β subunit also exist: β_1, expressed in skeletal muscle; β_2 and β_3, expressed in human brain tissue; and β_4, detected in aorta (Figure 5C). Additional forms of the β subunit may also be expressed, as indicated by two β-specific transcripts identified in skeletal muscle (Ruth et al., 1989).

Recently, a rabbit brain α_1 subunit, designated BI, was cloned and expressed (Mori et al., 1991). Not only does this subunit differ structurally from the α_{1D} subunit (Figure 2), but the biophysical and pharmacological properties of the Ca^{2+} channel, formed by coexpression of the BI subunit with the rabbit skeletal muscle α_{2a} and β_1 subunits, differ from those of the human neuronal $\alpha_{1D}\alpha_{2b}\beta_2$ recombinant channel. The BI-mediated Ca^{2+} channel activity is insensitive to both DHPs and ω-CgTx and inactivates rapidly compared with the α_{1D}–mediated activity. The expression in Xenopus oocytes of both the BI-mediated and α_{1D}–mediated I_{Ba} requires the coexpression of a β subunit. Thus, two structurally and pharmacologically distinct α_1 subunits expressed in neuronal tissues require a β subunit for functional Ca^{2+} channel activity in oocytes, in contrast to the α_1 subunits expressed in cardiac (Mikami et al., 1989) and smooth muscle (Biel et al., 1990).

The α_1 subunits expressed in both cardiac and lung tissues are likely encoded by the same gene (Biel et al., 1990). This gene encodes mRNAs that direct the synthesis of DHP-sensitive Ca^{2+} channels in Xenopus oocytes with macroscopic biophysical properties similar to the $\alpha_{1D}\alpha_{2b}\beta_2$ channel (Mikami et al., 1989; Biel et al., 1990). However, the human neuronal $\alpha_{1D}\alpha_{2b}\beta_2$ DHP-sensitive channel has a current–voltage relation that is shifted by approximately −20 mV, and its tail currents are markedly prolonged after Bay K 8644 application compared with the cardiac and lung channel

types. A comparison of the single-channel properties might further distinguish these different DHP-sensitive L-type Ca^{2+} channels.

The β_2 Subunit Stimulates DHP-Insensitive I_{Ba} in Xenopus Oocytes

Our results suggest that the α_2 and β subunits expressed in skeletal muscle (α_{2a} and β_1) differ structurally (Figure 3; Figure 4; Figure 5) and possibly functionally from the α_2 and β subunits expressed in brain tissue (α_{2b} and β_2). Xenopus oocytes coinjected with the rabbit skeletal muscle α_{2a} and β_1 mRNAs apparently do not display I_{Ba} upon depolarization (Mori et al., 1991). This is in contrast to our observation that oocytes injected with the human neuronal β_2 mRNA alone or coinjected with the β_2 and α_{2b} mRNAs display significant I_{Ba} upon depolarization. Coexpression of the α_{2b} subunit enhances the I_{Ba}, but α_{2b} mRNA shows no activity when injected alone.

The Ca^{2+} channel expressed in $\alpha_{2b}\beta_2$–injected oocytes has pharmacological and biophysical properties that resemble Xenopus oocyte endogenous voltage-dependent Ca^{2+} channels (Dascal et al., 1986). Similar to the skeletal muscle β_1 subunit (Ruth et al., 1989), the β_2 subunit lacks hydrophobic segments capable of forming transmembrane domains. Thus, it is unlikely that the β_2 subunit alone is forming an ion channel. It is more probable that a homologous α_1 subunit exists in oocytes comprising an endogenous Ca^{2+} channel and that the activity mediated by this α_1 subunit is enhanced by the expression of the β_2 subunit, similar to that observed for the α_{1D} and BI activities. Further information concerning the structure of the endogenous Xenopus oocyte Ca^{2+} channel is not yet available.

The Ca^{2+} channel stimulated by the presence of the β_2 subunit may contribute an inactivating, DHP-insensitive component of I_{Ba} to the total current in $\alpha_{1D}\alpha_{2b}\beta_2$–injected oocytes, especially when recorded from strongly negative holding potentials. Recordings made from $\alpha_{1D}\alpha_{2b}\beta_2$–injected oocytes at different holding potentials support this possibility and indicate that such contamination can be reduced, though not necessarily eliminated, by holding at −50 mV. The DHP-insensitive β_2–mediated current may account for the residual inactivating I_{Ba} detected in $\alpha_{1D}\alpha_{2b}\beta_2$–injected oocytes in the presence of nifedipine (Figure 6C).

ω-CgTx Interacts with the Neuronal DHP-Sensitive Ca^{2+} Channel

ω-CgTx blocks neuronal N-type Ca^{2+} channels irreversibly (Feldman et al., 1987; McCleskey et al., 1987). In contrast to this high affinity block, ω-CgTx blocks the $\alpha_{1D}\alpha_{2b}\beta_2$ channel reversibly with an affinity probably in the micromolar range, as indicated by the partial block with 10–15 μM ω-CgTx. Although preliminary experiments indicate that the $\alpha_{2b}\beta_2$–mediated channel may be inhibited by ω-CgTx, block of Bay K 8644–induced tail currents in $\alpha_{1D}\alpha_{2b}\beta_2$-injected oocytes demonstrates that ω-CgTx also interacts with the DHP-sensitive $\alpha_{1D}\alpha_{2b}\beta_2$ channel. Reversible block by ω-CgTx of L-type (Aosaki and Kasai, 1989), T-type (McCleskey et al., 1987), and a subclass of N-type (Plummer et al., 1989) Ca^{2+} channels has been reported. Furthermore, Ca^{2+}-dependent ATP release from elasmobranch electroplax synaptosomes is blocked reversibly by ω-CgTx with micromolar affinity (Yeager et al., 1987). It thus appears that variable affinity for ω-CgTx may be shared by several types of voltage-dependent Ca^{2+} channels. A weak block such as we have demonstrated for the $\alpha_{1D}\alpha_{2b}\beta_2$ L-type channel may account for the conflicting results reported in the literature concerning the ability of ω-CgTx to block neuronal L-type channels (McCleskey et al., 1987; Suzuki and Yoshioka, 1987; Aosaki and Kasai, 1989; Plummer et al., 1989).

Conclusion

The function of DHP-sensitive Ca^{2+} channels in skeletal and cardiac muscle has been extensively studied (Hess, 1990). In contrast, the role of the neuronal L-type Ca^{2+} channel is poorly understood (Miller, 1987). L-type Ca^{2+} channels may mediate the release of neurotransmitters from some types of neurons (Holz et al., 1988). However, functional analysis is difficult due to the mixed population of voltage-dependent Ca^{2+} channel subtypes in continuous cell lines as well as cells in primary tissues. For example, L-type Ca^{2+} channels contribute a minor fraction of the I_{Ba} detectable in the cell bodies of IMR32 cells (Carbone et al., 1990; unpublished data), PC12 cells (Plummer et al., 1989), certain sensory neurons (Aosaki and Kasai, 1989), and sympathetic neurons (Plummer et al., 1989; Jones and Jacobs, 1990). In contrast, L-type channels contribute substantial I_{Ba} in some populations of sensory neurons (Scroggs and Fox, 1991) and certain CNS neurons (Mogul and Fox, 1991; Regan et al., 1991).

Our characterization of a novel human neuronal voltage-dependent Ca^{2+} channel firmly establishes the existence of multiple subtypes of DHP-sensitive L-type Ca^{2+} channels. Furthermore, this human neuronal Ca^{2+} channel appears to have functional and pharmacological properties distinct from any other recombinant Ca^{2+} channel expressed to date. Together with the evidence for differentially processed mRNAs encoding three subunits of voltage-dependent Ca^{2+} channels, these results indicate that the molecular diversity of this ion channel class is much greater than previously proposed by traditional biophysical and pharmacological studies.

Experimental Procedures

Nomenclature

The following nomenclature is used for the α_1 gene family and the differentially processed α_2 and β transcripts. The structurally distinct human neuronal α_1 gene product described here is designated α_{1D} in accordance with its 96.3% deduced amino acid sequence identity to the rat brain class D sequence (Snutch et al., 1990). The α_2 gene product expressed in skeletal muscle is designated α_{2a}; the differentially processed α_2 transcript expressed in neuronal tissues is designated α_{2b}; the aorta α_2 tran-

script is designated α_{1c}. The β gene product expressed in skeletal muscle is designated β_1; the β transcript expressed in neuronal tissues is differentially processed to produce β_2 and β_3 transcripts. An additional β transcript expressed in aorta is designated β_4.

cDNA Libraries

Recombinant cDNA libraries were prepared, and individual cDNA clones were characterized essentially as previously described by Ellis et al. (1988). Unless otherwise noted, the nucleotide numbers in the text refer to cDNA coding sequence. For the isolation of human neuronal α_{1D} subunit cDNAs, RNA was isolated from the human neuroblastoma IMR32 cell line (ATCC #CCL127), which had been grown in 1.0 mM dibutyryl cAMP for 10 days. Four different cDNA libraries were constructed into the phage vector λgt11: oligo(dT)-primed double-stranded cDNA, 1–3 kb size fractionated by agarose gel electrophoresis; oligo(dT)-primed double-stranded cDNA, 3–9 kb size fractionated; random-primed double-stranded cDNA, >1.8 kb; and specifically primed (nucleotides 2417–2446 of α_{1D}) double-stranded cDNA, >1.5 kb. Human neuronal α_{2b} subunit cDNAs were isolated from a human basal ganglia cDNA library (ATCC #37433) and a human brain stem cDNA library (ATCC #37432). Human brain β_2 and β_3 subunit cDNAs were isolated from a human hippocampus cDNA library constructed in the λ phage vector λZAPII (Stratagene, La Jolla, CA, #936205).

Isolation of Recombinant cDNAs Encoding Different Ca^{2+} Channel Subunits

α_{1D} Subunit

Approximately 1 × 10^6 recombinants of the 1–3 kb library were screened with the rabbit skeletal muscle α_1 subunit cDNA (Ellis et al., 1988). Clone λα1.36 (nucleotides 2347–3771 of α_{1D}) was isolated and characterized, and the insert was used to screen the 3–9 kb library. Clone λα1.80 (nucleotides 1573–5958) was isolated and characterized, and the 3′ portion of the insert was used to screen the random-primed library from which clone λα1.163 (nucleotides 4690–7125) was isolated. The 5′ portion of α1.80 was subsequently used to screen the random-primed library, resulting in the isolation of clone λα1.144 (nucleotides −510 to 1921). The 5′ portion of α1.80 was then used to screen the specifically primed library from which clone λα1.136 (nucleotides 1117–2478) was isolated.

α_{2b} Subunit

Human genomic α_2 clones were isolated to use as α_2–specific probes of human neuronal cDNA libraries. A rabbit skeletal muscle α_{1A} cDNA fragment, clone SkMCaCHα2.2, comprising nucleotides 43–272 (Ellis et al., 1988), was used to identify and clone two α_2–specific, human genomic EcoRI fragments, HGCaCHα2.20 (3.5 kb) and HGCaCHα2.9 (3.0 kb). Restriction mapping and DNA sequencing revealed that HGCaCHα2.20 contains an 82 bp exon (nucleotides 96–177 of the human α_{2b} coding sequence) and that HGCaCHα2.9 contains 105 bp of an exon (nucleotides 178–282 of the coding sequence). These restriction fragments were used to screen the human basal ganglia cDNA library. HBCaCHα2.1 was isolated (nucleotides −6 to 1129) and used to screen the human brain stem cDNA library. Two clones were isolated, HBCaCHα2.5 (nucleotides −34 to 1128) and HBCaCHα2.8 (nucleotides 680–1528 followed by 1600 nucleotides of intervening sequence). HBCaCHα2.8 was used to rescreen the brain stem library and to isolate HBCaCHα2.11 (nucleotides 845–3566).

β_2 and β_3 Subunits

A rabbit skeletal muscle β_1 subunit cDNA fragment (Ellis et al., 1988; Ruth et al., 1989) was used to screen the human hippocampus cDNA library. Two clones, λβ1 and λβ4, were isolated that appear to encode alternative splice products of the human β subunit transcript expressed in the brain, β_2 and β_3, respectively. λβ1 begins at nucleotides 69 and extends 107 nucleotides beyond the translation stop codon, encoding 1367 nucleotides of coding sequence. λβ1 also contains a 448 nucleotide intron between nucleotides 1146 and 1147 of the coding sequence. λβ4 begins at nucleotide 246 of the coding sequence and diverges from β_2 at nucleotide 1333 as described in the Results. λβ1 was used to rescreen the hippocampus cDNA library from which clone λβ1.18 was isolated, characterized, and determined to encode nucleotides 1–325 of the β_2 coding sequence.

PCR Analysis

PCR analyses were performed essentially as described by Innis et al. (1990). IMR32 cell cytoplasmic RNA was prepared as described by Ausubel et al. (1988). For the analysis of the series of 5′ methionine codons in the α_{1D} cDNA, four oligonucleotide primers were synthesized (numbered in the 5′ to 3′ orientation): (1) nucleotides −39 to −18, beginning 39 nucleotides 5′ of the first methionine codon; (2) nucleotides 58–81; (3) nucleotides 164–187; and (4) nucleotides 314–291. The oligonucleotide pairs (1, 4), (2, 4), and (3, 4) were used to prime PCR assays of cytoplasmic RNA and human genomic DNA. PCR amplification of human genomic DNA and IMR32 cytoplasmic RNA with oligonucleotide pairs (2, 4) and (3, 4) gave the predicted size product (260 and 150 bp, respectively). The cytoplasmic RNA assayed with the pair (1, 4) gave the predicted size product (350 bp); a PCR product of genomic DNA primed with the pair (1, 4) was not detected. The lack of a PCR product primed with pair (1, 4) on genomic DNA suggested the possible presence of an intron between oligonucleotides 1 and 2 and indicated that the positive results with the RNAs could not be due to genomic DNA contamination of the RNA preparations. The cytoplasmic RNA PCR product of the (1, 4) oligonucleotide pair was cloned and sequenced.

Construction of Full-Length cDNAs

α_{1D} Subunit

pVDCCIII(A) was constructed using α1.144 (nucleotides −184 to 1222), α1.136 (nucleotides 1222–2157), α1.80 (nucleotides 2157–4784), and α1.163 (nucleotides 4784–7125). PCR analysis of the α_{1D} transcript revealed that α1.80 contained a 148 nucleotide deletion (nucleotides 2474–2621). To correct this deletion, PCR was performed on IMR32 RNA, and the AccI–BglII fragment (nucleotides 2254–3380) was isolated and used to replace the α1.80 fragment.

α_{2b} Subunit

pHBCaCHα$_{2b}$(A) was constructed using HBCaCHα2.5 (nucleotides −34 to 1027) and HBCaCHα2.11 (nucleotides 1027–3566).

β_2 Subunit

To construct pHBCaCHβ$_{2b}$.RBS(A), the 448 nucleotide intron of λβ1 first was deleted via site-directed mutagenesis (Sambrook et al., 1989). λβ1 was subcloned into M13mp19. The mutagenic oligonucleotide was the sense strand of β_2 encoding nucleotides 1128–1165. The final construct was designated pβ1(−). pHBCaCHβ$_{2b}$.RBS(A) then was constructed using λβ1.18 (nucleotides 1–282) and pβ1(−) (nucleotides 282–1547). The 5′ untranslated sequence in λβ1.18 was replaced with an efficient ribosomal-binding site so that the sequence reads 5′-GAATTC (EcoRI) ACCACC (ribosomal-binding site) ATG (start codon) ... −3′. Each α_{1D}, α_{2b}, and β_2 full-length construct was subcloned into pcDNA1 (Invitrogen, San Diego, CA).

Expression Studies in Xenopus Oocytes

In vitro transcripts of human neuronal α_{1D}, α_{2b}, and β_2 subunit cDNAs were synthesized according to the instructions of the mCAP mRNA Capping Kit (Stratagene, La Jolla, CA, #200350). Each plasmid first was linearized by restriction digestion: pVDCCIII(A) with XhoI, pHBCaCHα$_{2b}$(A) with XhoI, and pHBCaCHβ$_{2b}$.RBS(A) with EcoRV. T7 RNA polymerase was used to transcribe each cDNA. Xenopus laevis oocytes were dissociated and defolliculated by collagenase treatment and maintained in 100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES (pH 7.6), 20 μg/ml ampicillin, and 25 μg/ml streptomycin at 19°C–25°C for 2–5 days after injection and prior to recording. Oocytes were injected with 6 ng of each in vitro synthesized mRNA species per cell in a volume of 50 nl and were assayed by the two-electrode voltage-clamp method (Dascal, 1987) using the pClamp (Axon Instruments) software package in conjunction with a Labmaster 125 kHz data acquisition interface (Scientific Solutions) to generate voltage commands and to acquire and analyze data. Current signals were digitized at 1–5 kHz and filtered appropriately. I_{Ba}

was recorded in a solution intended to minimize currents carried through K^+, Cl^-, or Na^+ channels (Snutch et al., 1990): 40 mM $BaCl_2$, 36 mM tetraethylammonium chloride, 2 mM KCl, 5 mM 4-aminopyridine, 0.15 mM niflumic acid, 5 mM HEPES (pH 7.6). Currents were leak subtracted by the P/n method provided in pClamp, where n was −4 or −6. Drugs were applied directly into the 60 μl bath while the perfusion pump was turned off. Bay K 8644 and nifedipine were prepared fresh from stock solutions (in dimethyl sulfoxide) and diluted into the bath solution. The dimethyl sulfoxide concentration of the final drug solutions in contact with the cell never exceeded 0.1%. Control experiments showed that 0.1% dimethyl sulfoxide had no effect on membrane currents. ω-CgTx was prepared in a 15 mM $BaCl_2$ bath solution plus 0.1% cytochrome C (Sigma) (Feldman et al., 1987) to serve as a carrier protein. Control experiments showed that cytochrome C had no effect on currents. Before and during ω-CgTx application, test pulses were recorded at 20 s intervals from the holding potential (−90 mV or −50 mV) to the peak I_{Ba} (−10 mV to 10 mV). To reduce the inhibition of ω-CgTx binding by divalent cations (McCleskey et al., 1987), recordings were made in 15 mM $BaCl_2$, 73.5 mM tetraethylammonium chloride, and the remaining ingredients identical to the 40 mM Ba^{2+} recording solution.

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GenBank Accession Numbers

The nucleotide sequences of the human α_{1D}, α_{2b}, and β_3 cDNAs will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under the accession numbers M76558 (α_{1D}), M76559 (α_{2b}), and M76560 (β_3).

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(54) **DNA ENCODING HUMAN ALPHA1G-C T-TYPE CALCIUM CHANNEL**

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(57) **ABSTRACT**

A DNA molecule encoding a novel isoform of the human T-type low voltage activated calcium channel (alpha1G-c) has been cloned and characterized. The biological and structural properties of this protein is disclosed, as is the amino acid and nucleotide sequence.

7 Claims, 9 Drawing Sheets-

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FIG. 1A

SEQ.ID.NO.3. Human calcium channel alpha1G-c sequence of the coding sequence (6822 bp includes the TGA).

ATGGACGAGGAGGAGGATGGAGCGGGCGCCGAGGAGTCGGGACAGCCCCGGAGCTTCAT
GCGGCTCAACGACCTGTCGGGGGCCGGGGGCCGGCCGGGGCCGGGGTCAGCAGAAAAGG
ACCCGGGCAGCGCGGACTCCGAGGCGGAGGGGCTGCCGTACCCGGCGCTGGCCCCGGTG
GTTTTCTTCTACTTGAGCCAGGACAGCCGCCCGCGGAGCTGGTGTCTCCGCACGGTCTG
TAACCCCTGGTTTGAGCGCATCAGCATGTTGGTCATCCTTCTCAACTGCGTGACCCTGG
GCATGTTCCGGCCATGCGAGGACATCGCCTGTGACTCCCAGCGCTGCCGGATCCTGCAG
GCCTTTGATGACTTCATCTTTGCCTTCTTTGCCGTGGAGATGGTGGTGAAGATGGTGGC
CTTGGGCATCTTTGGGAAAAAGTGTTACCTGGGAGACACTTGGAACCGGCTTGACTTTT
TCATCGTCATCGCAGGGATGCTGGAGTACTCGCTGGACCTGCAGAACGTCAGCTTCTCA
GCTGTCAGGACAGTCCGTGTGCTGCGACCGCTCAGGGCCATTAACCGGGTGCCCAGCAT
GCGCATCCTTGTCACGTTGCTGCTGGATACGCTGCCCATGCTGGGCAACGTCCTGCTGC
TCTGCTTCTTCGTCTTCTTCATCTTCGGCATCGTCGGCGTCCAGCTGTGGGCAGGGCTG
CTTCGGAACCGATGCTTCCTACCTGAGAATTTCAGCCTCCCCCTGAGCGTGGACCTGGA
GCGCTATTACCAGACAGAGAACGAGGATGAGAGCCCCTTCATCTGCTCCCAGCCACGCG
AGAACGGCATGCGGTCCTGCAGAAGCGTGCCCACGCTGCGCGGGGACGGGGGCGGTGGC
CCACCTTGCGGTCTGGACTATGAGGCCTACAACAGCTCCAGCAACACCACCTGTGTCAA
CTGGAACCAGTACTACACCAACTGCTCAGCGGGGGAGCACAACCCCTTCAAGGGCGCCA
TCAACTTTGACAACATTGGCTATGCCTGGATCGCCATCTTCCAGGTCATCACGCTGGAG
GGCTGGGTCGACATCATGTACTTTGTGATGGATGCTCATTCCTTCTACAATTTCATCTA
CTTCATCCTCCTCATCATCGTGGGCTCCTTCTTCATGATCAACCTGTGCCTGGTGGTGA
TTGCCACGCAGTTCTCAGAGACCAAGCAGCGGGAAAGCCAGCTGATGCGGGAGCAGCGT
GTGCGGTTCCTGTCCAACGCCAGCACCCTGGCTAGCTTCTCTGAGCCCGGCAGCTGCTA
TGAGGAGCTGCTCAAGTACCTGGTGTACATCCTTCGTAAGGCAGCCCGCAGGCTGGCTC
AGGTCTCTCGGGCAGCAGGTGTGCGGGTTGGGCTGCTCAGCAGCCCAGCACCCCTCGGG
GGCCAGGAGACCCAGCCCAGCAGCAGCTGCTCTCGCTCCCACCGCCGCCTATCCGTCCA
CCACCTGGTGCACCACCACCACCACCATCACCACCACTACCACCTGGGCAATGGGACGC
TCAGGGCCCCCCCGGGCCAGCCCGGAGATCCAGGACAGGGATGCCAATGGGTCCCGCAGG
CTCATGCTGCCACCACCCTCGACGCCTGCCCTCTCCGGGGCCCCCCCTGGTGGCGCAGA
GTCTGTGCACAGCTTCTACCATGCCGACTGCCACTTAGAGCCAGTCCGCTGCCAGGCGC
CCCCTCCCAGGTCCCCATCTGAGGCATCCGGCAGGACTGTGGGCAGCGGGAAGGTGTAT
CCCACCGTGCACACCAGCCCTCCACCGGAGACGCTGAAGGAGAAGGCACTAGTAGAGGT
GGCTGCCAGCTCTGGGCCCCCAACCCTCACCAGCCTCAACATCCCACCCGGGCCCTACA
GCTCCATGCACAAGCTGCTGGAGACACAGAGTACAGGTGCCTGCCAAAGCTCTTGCAAG
ATCTCCAGCCCTTGCTTGAAAGCAGACAGTGGAGCCTGTGGTCCAGACAGCTGCCCCTA
CTGTGCCCGGGCCGGGGCAGGGGAGGTGGAGCTCGCCGACCGTGAAATGCCTGACTCAG
ACAGCGAGGCAGTTTATGAGTTCACACAGGATGCCCAGCACAGCGACCTCCGGGACCCC
CACAGCCGGCGGCAACGGAGCCTGGGCCCAGATGCAGAGCCCAGCTCTGTGCTGGCCTT
CTGGAGGCTAATCTGTGACACCTTCCGAAAGATTGTGGACAGCAAGTACTTTGGCCGGG
GAATCATGATCGCCATCCTGGTCAACACACTCAGCATGGGCATCGAATACCACGAGCAG
CCCGAGGAGCTTACCAACGCCCTAGAAATCAGCAACATCGTCTTCACCAGCCTCTTTGC
CCTGGAGATGCTGCTGAAGCTGCTTGTGTATGGTCCCTTTGGCTACATCAAGAATCCCT
ACAACATCTTCGATGGTGTCATTGTGGTCATCAGCGTGTGGGAGATCGTGGGCCAGCAG
GGGGGCGGCCTGTCGGTGCTGCGGACCTTCCGCCTGATGCGTGTGCTGAAGCTGGTGCG
CTTCCTGCCGGCGCTGCAGCGGCAGCTGGTGGTGCTCATGAAGACCATGGACAACGTGG
CCACCTTCTGCATGCTGCTTATGCTCTTCATCTTCATCTTCAGCATCCTGGGCATGCAT
CTCTTCGGCTGCAAGTTTGCCTCTGAGCGGGATGGGGACACCCTGCCAGACCGGAAGAA
TTTTGACTCCTTGCTCTGGGCCATCGTCACTGTCTTTCAGATCCTGAC

FIG. 1B

CCAGGAGGACTGGAACAAAGTCCTCTACAATGGTATGGCCTCCACGTCGTCCTGGGCGG
CCCTTTATTTCATTGCCCTCATGACCTTCGGCAACTACGTGCTCTTCAATTTGCTGGTC
GCCATTCTGGTGGAGGGCTTCCAGGCGGAGGAAATCAGCAAACGGGAAGATGCGAGTGG
ACAGTTAAGCTGTATTCAGCTGCCTGTCGACTCCCAGGGGGGAGATGCCAACAAGTCCG
AATCAGAGCCCGATTTCTTCTCACCCAGCCTGGATGGTGATGGGGACAGGAAGAAGTGC
TTGGCCTTGGTGTCCCTGGGAGAGCACCCGGAGCTGCGGAAGAGCCTGCTGCCGCCTCT
CATCATCCACACGGCCGCCACACCCATGTCGCTGCCCAAGAGCACCAGCACGGGCCTGG
GCGAGGCGCTGGGCCCTGCGTCGCGCCGCACCAGCAGCAGCGGGTCGGCAGAGCCTGGG
GCGGCCCACGAGATGAAGTCACCGCCCAGCGCCCGCAGCTCTCCGCACAGCCCCTGGAG
CGCTGCAAGCAGCTGGACCAGCAGGCGCTCCAGCCGGAACAGCCTCGGCCGTGCACCCA
GCCTGAAGCGGAGAAGCCCAAGTGGAGAGCGGCGGTCCCTGTTGTCGGGAGAAGGCCAG
GAGAGCCAGGATGAAGAGGAGAGCTCAGAAGAGGAGCGGGCCAGCCCTGCGGGCAGTGA
CCATCGCCACAGGGGGTCCCTGGAGCGGGAGGCCAAGAGTTCCTTTGACCTGCCAGACA
CACTGCAGGTGCCAGGGCTGCATCGCACTGCCAGTGGCCGAGGGTCTGCTTCTGAGCAC
CAGGACTGCAATGGCAAGTCGGCTTCAGGGCGCCTGGCCCGGGCCCTGCGGCCTGATGA
CCCCCCACTGGATGGGGATGACGCCGATGACGAGGGCAACCTGAGCAAAGGGGAACGGG
TCCGCGCGTGGATCCGAGCCCGACTCCCTGCCTGCTGCCTCGAGCGAGACTCCTGGTCA
GCCTACATCTTCCCTCCTCAGTCCAGGTTCCGCCTCCTGTGTCACCGGATCATCACCCA
CAAGATGTTCGACCACGTGGTCCTTGTCATCATCTTCCTTAACTGCATCACCATCGCCA
TGGAGCGCCCCAAAATTGACCCCCACAGCGCTGAACGCATCTTCCTGACCCTCTCCAAT
TACATCTTCACCGCAGTCTTTCTGGCTGAAATGACAGTGAAGGTGGTGGCACTGGGCTG
GTGCTTCGGGGAGCAGGCGTACCTGCGGAGCAGTTGGAACGTGCTGGACGGGCTGTTGG
TGCTCATCTCCGTCATCGACATTCTGGTGTCCATGGTCTCTGACAGCGGCACCAAGATC
CTGGGCATGCTGAGGGTGCTGCGGCTGCTGCGGACCCTGCGCCCGCTCAGGGTGATCAG
CCGGGCGCAGGGGCTGAAGCTGGTGGTGGAGACGCTGATGTCCTCACTGAAACCCATCG
GCAACATTGTAGTCATCTGCTGTGCCTTCTTCATCATTTTCGGCATCTTGGGGGTGCAG
CTCTTCAAAGGGAAGTTTTTCGTGTGCCAGGGCGAGGATACCAGGAACATCACCAATAA
ATCGGACTGTGCCGAGGCCAGTTACCGGTGGGTCCGGCACAAGTACAACTTTGACAACC
TTGGCCAGGCCCTGATGTCCCTGTTCGTTTTGGCCTCCAAGGATGGTTGGGTGGACATC
ATGTACGATGGGCTGGATGCTGTGGGCGTGGACCAGCAGCCCATCATGAACCACAACCC
CTGGATGCTGCTGTACTTCATCTCGTTCCTGCTCATTGTGGCCTTCTTTGTCCTGAACA
TGTTTGTGGGTGTGGTGGTGGAGAACTTCCACAAGTGTCGGCAGCACCAGGAGGAAGAG
GAGGCCCGGCGGCGGGAGGAGAAGCGCCTACGAAGACTGGAGAAAAAGAGAAGGAGTAA
GGAGAAGCAGATGGCTGAAGCCCAGTGCAAACCTTACTACTCCGACTACTCCCGCTTCC
GGCTCCTCGTCCACCACTTGTGCACCAGCCACTACCTGGACCTCTTCATCACAGGTGTC
ATCGGGCTGAACGTGGTCACCATGGCCATGGAGCACTACCAGCAGCCCCAGATTCTGGA
TGAGGCTCTGAAGATCTGCAACTACATCTTCACTGTCATCTTTGTCTTGGAGTCAGTTT
TCAAACTTGTGGCCTTTGGTTTCCGTCGGTTCTTCCAGGACAGGTGGAACCAGCTGGAC
CTGGCCATTGTGCTGCTGTCCATCATGGGCATCACGCTGGAGGAAATCGAGGTCAACGC
CTCGCTGCCCATCAACCCCACCATCATCCGCATCATGAGGGTGCTGCGCATTGCCCGAG
TGCTGAAGCTGCTGAAGATGGCTGTGGGCATGCGGGCGCTGCTGGACACGGTGATGCAG
GCCCTGCCCCAGGTGGGGAACCTGGGACTTCTCTTCATGTTGTTGTTTTTCATCTTTGC
AGCTCTGGGCGTGGAGCTCTTTGGAGACCTGGAGTGTGACGAGACACACCCCTGTGAGG
GCCTGGGCCGTCATGCCACCTTTCGGAACTTTGGCATGGCCTTCCTAACCCTCTTCCGA
GTCTCCACAGGTGACAATTGGAATGGCATTATGAAGGACACCCTCCGGGACTGTGACCA
GGAGTCCACCTGCTACAACACGGTCATCTCGCCTATCTACTTTGTGTCCTTCGTGCTGA
CGGCCCAGTTCGTGCTAGTCAACGTGGTGATCGCCGTGCTGATGAAGCACCTGGAGGAG
AGCAACAAGGAGGCCAAGGAGGAGGCCGAGCTAGAGGCTGAGCTGGAGCTGGAGATGAA
GACCCTCAGCCCCCAGCCCCACTCGCCACTGGGCAGCCCCTTCCTCTGGCCTGGGGTCG
AGGGCCCCGACAGCCCCGACAGCCCCAAGCCTGGGGCTCTGCACCCAGCGGCCCACGCG
AGATCAGCCTCCCACTTTTCCCTGGAGCACCCCACGATGCAGCCCCACCCCACGGAGCT
GCCAGGACCAGACTTACTGACTGTGCGGAAGTCTGGGGTCAGCCGAACGCACTCTCTGC
CCAATGACAGCTACATGTGTCGGCATGGGAGCACTGCCGAGGGGCCCCTGGGACACAGG
GGCTGGGGGC

FIG. 1C

TCCCCAAAGCTCAGTCAGGCTCCGTCTTGTCCGTTCACTCCCAGCCAGCAGATACC
AGCTACATCCTGCAGCTTCCCAAAGATGCACCTCATCTGCTCCAGCCCCACAGCGC
CCCAACCTGGGGCACCATCCCCAAACTGCCCCCACCAGGACGCTCCCCTTTGGCTC
AGAGGCCACTCAGGCGCCAGGCAGCAATAAGGACTGACTCCTTGGACGTTCAGGGT
CTGGGCAGCCGGGAAGACCTGCTGGCAGAGGTGAGTGGGCCCTCCCCGCCCCTGGC
CCGGGCCTACTCTTTCTGGGGCCAGTCAAGTACCCAGGCACAGCAGCACTCCCGCA
GCCACAGCAAGATCTCCAAGCACATGACCCCGCCAGCCCCTTGCCCAGGCCCAGAA
CCCAACTGGGGCAAGGGCCCTCCAGAGACCAGAAGCAGCTTAGAGTTGGACACGGA
GCTGAGCTGGATTTCAGGAGACCTCCTGCCCCCTGGCGGCCAGGAGGAGCCCCCAT
CCCCACGGGACCTGAAGAAGTGCTACAGCGTGGAGGCCCAGAGCTGCCAGCGCCGG
CCTACGTCCTGGCTGGATGAGCAGAGGAGACACTCTATCGCCGTCAGCTGCCTGGA
CAGCGGCTCCCAACCCCACCTGGGCACAGACCCCTCTAACCTTGGGGGCCAGCCTC
TTGGGGGGCCCGGGAGCCGGCCCAAGAAAAAACTCAGCCCGCCTAGTATCACCATA
GACCCCCCCGAGAGCCAAGGTCCTCGGACCCCGCCCAGCCCTGGTATCTGCCTCCG
GAGGAGGGCTCCGTCCAGCGACTCCAAGGATCCCTTGGCCTCTGGCCCCCCTGACA
GCATGGCTGCCTCGCCCTCCCCAAAGAAAGATGTGCTGAGTCTCTCCGGTTTATCC
TCTGACCCAGCAGACCTGGACCCCTGA

FIG. 2A

SEQ.ID.NO.4. The nucleotide sequence of human calcium channel alpha1G-c is shown including 522 bp 5' UT and 397 bp 3'UT.

CCGGGTCGACCCACGCGTCCGGATCCCTCCTCCCCTCCCCCGCCGCCTGGCGCGGAG
CCGGGACGATGCTGACCCCTTAGATCCGGCTCCAGCTGCGCCGCGGGAAGAGGGGGC
GCCCCTCCCCGGACCCCCGCCCTCCGCCGCTGCCCCCCTTTTCGTTCGCCCTCTCGG
GGCGGCTTCGCCGAAGGTAGCGCCGAATCCGGCAACCGGAGCCTGGGCGCGAAGCGA
AGAAGCCGGAACAAAGTGAGGGGGAGCCGGCCGGCTGGCCCGGGAAGCCCCAGGGGC
GCAGGGGAAGCGGGACTCGCGCCGGGCGGGGTTTCCCTGCGCCCCGGCGCCCCGCGG
GCAGCATGCCCCTGCGGGCAGGGGGAGCTGGGCTGAACTGGCCCTCCCGGGGGCTCA
GCTTGCGCCCTAGAGCCCACCAGATGTGCCCCCGCCGGGGCCCCCGGGTTGCGTGAG
GACACCTCCTCTGAGGGGCGCCGCTTGCCCCTCTCCGGATCGCCCGGGGCCCCGGCT
GGCCAGAGGATGGACGAGGAGGAGGATGGAGCGGGCGCCGAGGAGTCGGGACAGCCC
CGGAGCTTCATGCGGCTCAACGACCTGTCGGGGGCCGGGGGCCGGCCGGGGCCGGGG
TCAGCAGAAAAGGACCCGGGCAGCGCGGACTCCGAGGCGGAGGGGCTGCCGTACCCG
GCGCTGGCCCCGGTGGTTTTCTTCTACTTGAGCCAGGACAGCCGCCCGCGGAGCTGG
TGTCTCCGCACGGTCTGTAACCCCTGGTTTGAGCGCATCAGCATGTTGGTCATCCTT
CTCAACTGCGTGACCCTGGGCATGTTCCGGCCATGCGAGGACATCGCCTGTGACTCC
CAGCGCTGCCGGATCCTGCAGGCCTTTGATGACTTCATCTTTGCCTTCTTTGCCGTG
GAGATGGTGGTGAAGATGGTGGCCTTGGGCATCTTTGGGAAAAAGTGTTACCTGGGA
GACACTTGGAACCGGCTTGACTTTTTCATCGTCATCGCAGGGATGCTGGAGTACTCG
CTGGACCTGCAGAACGTCAGCTTCTCAGCTGTCAGGACAGTCCGTGTGCTGCGACCG
CTCAGGGCCATTAACCGGGTGCCCAGCATGCGCATCCTTGTCACGTTGCTGCTGGAT
ACGCTGCCCATGCTGGGCAACGTCCTGCTGCTCTGCTTCTTCGTCTTCTTCATCTTC
GGCATCGTCGGCGTCCAGCTGTGGGCAGGGCTGCTTCGGAACCGATGCTTCCTACCT
GAGAATTTCAGCCTCCCCCTGAGCGTGGACCTGGAGCGCTATTACCAGACAGAGAAC
GAGGATGAGAGCCCCTTCATCTGCTCCCAGCCACGCGAGAACGGCATGCGGTCCTGC
AGAAGCGTGCCCACGCTGCGCGGGGACGGGGGCGGTGGCCCACCTTGCGGTCTGGAC
TATGAGGCCTACAACAGCTCCAGCAACACCACCTGTGTCAACTGGAACCAGTACTAC
ACCAACTGCTCAGCGGGGGAGCACAACCCCTTCAAGGGCGCCATCAACTTTGACAAC
ATTGGCTATGCCTGGATCGCCATCTTCCAGGTCATCACGCTGGAGGGCTGGGTCGAC
ATCATGTACTTTGTGATGGATGCTCATTCCTTCTACAATTTCATCTACTTCATCCTC
CTCATCATCGTGGGCTCCTTCTTCATGATCAACCTGTGCCTGGTGGTGATTGCCACG
CAGTTCTCAGAGACCAAGCAGCGGGAAAGCCAGCTGATGCGGGAGCAGCGTGTGCGG
TTCCTGTCCAACGCCAGCACCCTGGCTAGCTTCTCTGAGCCCGGCAGCTGCTATGAG
GAGCTGCTCAAGTACCTGGTGTACATCCTTCGTAAGGCAGCCCGCAGGCTGGCTCAG
GTCTCTCGGGCAGCAGGTGTGCGGGTTGGGCTGCTCAGCAGCCCAGCACCCCTCGGG
GGCCAGGAGACCCAGCCCAGCAGCAGCTGCTCTCGCTCCCACCGCCGCCTATCCGTC
CACCACCTGGTGCACCACCACCACCACCATCACCACCACTACCACCTGGGCAATGGG
ACGCTCAGGGCCCCCCGGGCCAGCCCGGAGATCCAGGACAGGGATGCCAATGGGTCC
CGCAGGCTCATGCTGCCACCACCCTCGACGCCTGCCCTCTCCGGGGCCCCCCCTGGT
GGCGCAGAGTCTGTGCACAGCTTCTACCATGCCGACTGCCACTTAGAGCCAGTCCGC
TGCCAGGCGCCCCCTCCCAGGTCCCCATCTGAGGCATCCGGCAGGACTGTGGGCAGC
GGGAAGGTGTATCCCACCGTGCACACCAGCCCTCCACCGGAGACGCTGAAGGAGAAG
GCACTAGTAGAGGTGGCTGCCAGCTCTGGGCCCCCAACCCTCACCAGCCTCAACATC
CCACCCGGGCCCTACAGCTCCATGCACAAGCTGCTGGAGACACAGAGTACAGGTGCC
TGCCAAAGCTCTTGCAAGATCTCCAGCCCTTGCTTGAAAGCAGACAGTGGAGCCTGT
GGTCCAGACAGCTGCCCCTACTGTGCCCGGGCCGGGGCAGGGGAGGTGGAGCTCGCC
GACCGTGAAATGCCTGACTCAGACAGCGAGGCAGTTTATGAGTTCACACAGGATGCC
CAGCACAGCGACCTCCGGGACCCCCACAGCCGGCGGCAACGGAGCCTGGGCCCAGAT
GCAGAGCCCAGC

FIG. 2B

TCTGTGCTGGCCTTCTGGAGGCTAATCTGTGACACCTTCCGAAAGATTGTGGACAGCAAG
TACTTTGGCCGGGGAATCATGATCGCCATCCTGGTCAACACACTCAGCATGGGCATCGAA
TACCACGAGCAGCCCGAGGAGCTTACCAACGCCCTAGAAATCAGCAACATCGTCTTCACC
AGCCTCTTTGCCCTGGAGATGCTGCTGAAGCTGCTTGTGTATGGTCCCTTTGGCTACATC
AAGAATCCCTACAACATCTTCGATGGTGTCATTGTGGTCATCAGCGTGTGGGAGATCGTG
GGCCAGCAGGGGGGCGGCCTGTCGGTGCTGCGGACCTTCCGCCTGATGCGTGTGCTGAAG
CTGGTGCGCTTCCTGCCGGCGCTGCAGCGGCAGCTGGTGGTGCTCATGAAGACCATGGAC
AACGTGGCCACCTTCTGCATGCTGCTTATGCTCTTCATCTTCATCTTCAGCATCCTGGGC
ATGCATCTCTTCGGCTGCAAGTTTGCCTCTGAGCGGGATGGGGACACCCTGCCAGACCGG
AAGAATTTTGACTCCTTGCTCTGGGCCATCGTCACTGTCTTTCAGATCCTGACCCAGGAG
GACTGGAACAAAGTCCTCTACAATGGTATGGCCTCCACGTCGTCCTGGGCGGCCCTTTAT
TTCATTGCCCTCATGACCTTCGGCAACTACGTGCTCTTCAATTTGCTGGTCGCCATTCTG
GTGGAGGGCTTCCAGGCGGAGGAAATCAGCAAACGGGAAGATGCGAGTGGACAGTTAAGC
TGTATTCAGCTGCCTGTCGACTCCCAGGGGGGAGATGCCAACAAGTCCGAATCAGAGCCC
GATTTCTTCTCACCCAGCCTGGATGGTGATGGGGACAGGAAGAAGTGCTTGGCCTTGGTG
TCCCTGGGAGAGCACCCGGAGCTGCGGAAGAGCCTGCTGCCGCCTCTCATCATCCACACG
GCCGCCACACCCATGTCGCTGCCCAAGAGCACCAGCACGGGCCTGGGCGAGGCGCTGGGC
CCTGCGTCGCGCCGCACCAGCAGCAGCGGGTCGGCAGAGCCTGGGGCGGCCCACGAGATG
AAGTCACCGCCCAGCGCCCGCAGCTCTCCGCACAGCCCCTGGAGCGCTGCAAGCAGCTGG
ACCAGCAGGCGCTCCAGCCGGAACAGCCTCGGCCGTGCACCCAGCCTGAAGCGGAGAAGC
CCAAGTGGAGAGCGGCGGTCCCTGTTGTCGGGAGAAGGCCAGGAGAGCCAGGATGAAGAG
GAGAGCTCAGAAGAGGAGCGGGCCAGCCCTGCGGGCAGTGACCATCGCCACAGGGGGTCC
CTGGAGCGGGAGGCCAAGAGTTCCTTTGACCTGCCAGACACACTGCAGGTGCCAGGGCTG
CATCGCACTGCCAGTGGCCGAGGGTCTGCTTCTGAGCACCAGGACTGCAATGGCAAGTCG
GCTTCAGGGCGCCTGGCCCGGGCCCTGCGGCCTGATGACCCCCCACTGGATGGGGATGAC
GCCGATGACGAGGGCAACCTGAGCAAAGGGGAACGGGTCCGCGCGTGGATCCGAGCCCGA
CTCCCTGCCTGCTGCCTCGAGCGAGACTCCTGGTCAGCCTACATCTTCCCTCCTCAGTCC
AGGTTCCGCCTCCTGTGTCACCGGATCATCACCCACAAGATGTTCGACCACGTGGTCCTT
GTCATCATCTTCCTTAACTGCATCACCATCGCCATGGAGCGCCCCAAAATTGACCCCCAC
AGCGCTGAACGCATCTTCCTGACCCTCTCCAATTACATCTTCACCGCAGTCTTTCTGGCT
GAAATGACAGTGAAGGTGGTGGCACTGGGCTGGTGCTTCGGGGAGCAGGCGTACCTGCGG
AGCAGTTGGAACGTGCTGGACGGGCTGTTGGTGCTCATCTCCGTCATCGACATTCTGGTG
TCCATGGTCTCTGACAGCGGCACCAAGATCCTGGGCATGCTGAGGGTGCTGCGGCTGCTG
CGGACCCTGCGCCCGCTCAGGGTGATCAGCCGGGCGCAGGGGCTGAAGCTGGTGGTGGAG
ACGCTGATGTCCTCACTGAAACCCATCGGCAACATTGTAGTCATCTGCTGTGCCTTCTTC
ATCATTTTCGGCATCTTGGGGGTGCAGCTCTTCAAAGGGAAGTTTTTCGTGTGCCAGGGC
GAGGATACCAGGAACATCACCAATAAATCGGACTGTGCCGAGGCCAGTTACCGGTGGGTC
CGGCACAAGTACAACTTTGACAACCTTGGCCAGGCCCTGATGTCCCTGTTCGTTTTGGCC
TCCAAGGATGGTTGGGTGGACATCATGTACGATGGGCTGGATGCTGTGGGCGTGGACCAG
CAGCCCATCATGAACCACAACCCCTGGATGCTGCTGTACTTCATCTCGTTCCTGCTCATT
GTGGCCTTCTTTGTCCTGAACATGTTTGTGGGTGTGGTGGTGGAGAACTTCCACAAGTGT
CGGCAGCACCAGGAGGAAGAGGAGGCCCGGCGGCGGGAGGAGAAGCGCCTACGAAGACTG
GAGAAAAAGAGAAGGAGTAAGGAGAAGCAGATGGCTGAAGCCCAGTGCAAACCTTACTAC
TCCGACTACTCCCGCTTCCGGCTCCTCGTCCACCACTTGTGCACCAGCCACTACCTGGAC
CTCTTCATCACAGGTGTCATCGGGCTGAACGTGGTCACCATGGCCATGGAGCACTACCAG
CAGCCCCAGATTCTGGATGAGGCTCTGAAGATCTGCAACTACATCTTCACTGTCATCTTT
GTCTTGGAGTCAGTTTTCAAACTTGTGGCCTTTGGTTTCCGTCGGTTCTTCCAGGACAGG
TGGAACCAGCTGGACCTGGCCATTGTGCTGCTGTCCATCATGGGCATCACGCTGGAGGAA
ATCGAGGTCAACGCCTCGCTGCCCATCAACCCCACCATCATCCGCATCATGAGGGTGCTG
CGCATTGCCCGAGTGCTGAAGCTGCTGAAGATGGCTGTGGGCATGCGGGCGCTGCTGGAC
ACGGTGATGCAGGCCCTGCCCCAGGTGGGGAACCTGGGACTTCTCTTCATGTTGTTGTTT
TTCATCTTTGCAGCTCTGGGCGTGGAGCTCTTTGGAGACCTGGAGTGTGACGAGACACAC
CCCTGTGAGGGCCTGGGCCGT

FIG. 2C

CATGCCACCTTTCGGAACTTTGGCATGGCCTTCCTAACCCTCTTCCGAGTCTCCACA
GGTGACAATTGGAATGGCATTATGAAGGACACCCTCCGGGACTGTGACCAGGAGTCC
ACCTGCTACAACACGGTCATCTCGCCTATCTACTTTGTGTCCTTCGTGCTGACGGCC
CAGTTCGTGCTAGTCAACGTGGTGATCGCCGTGCTGATGAAGCACCTGGAGGAGAGC
AACAAGGAGGCCAAGGAGGAGGCCGAGCTAGAGGCTGAGCTGGAGCTGGAGATGAAG
ACCCTCAGCCCCCAGCCCCACTCGCCACTGGGCAGCCCCTTCCTCTGGCCTGGGGTC
GAGGGCCCCGACAGCCCCGACAGCCCCAAGCCTGGGGCTCTGCACCCAGCGGCCCAC
GCGAGATCAGCCTCCCACTTTTCCCTGGAGCACCCCACGATGCAGCCCCACCCCACG
GAGCTGCCAGGACCAGACTTACTGACTGTGCGGAAGTCTGGGGTCAGCCGAACGCAC
TCTCTGCCCAATGACAGCTACATGTGTCGGCATGGGAGCACTGCCGAGGGGCCCCTG
GGACACAGGGGCTGGGGGCTCCCCAAAGCTCAGTCAGGCTCCGTCTTGTCCGTTCAC
TCCCAGCCAGCAGATACCAGCTACATCCTGCAGCTTCCCAAAGATGCACCTCATCTG
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CGCTCCCCTTTGGCTCAGAGGCCACTCAGGCGCCAGGCAGCAATAAGGACTGACTCC
TTGGACGTTCAGGGTCTGGGCAGCCGGGAAGACCTGCTGGCAGAGGTGAGTGGGCCC
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CAGCACTCCCGCAGCCACAGCAAGATCTCCAAGCACATGACCCCGCCAGCCCCTTGC
CCAGGCCCAGAACCCAACTGGGGCAAGGGCCCTCCAGAGACCAGAAGCAGCTTAGAG
TTGGACACGGAGCTGAGCTGGATTTCAGGAGACCTCCTGCCCCCTGGCGGCCAGGAG
GAGCCCCCATCCCCACGGGACCTGAAGAAGTGCTACAGCGTGGAGGCCCAGAGCTGC
CAGCGCCGGCCTACGTCCTGGCTGGATGAGCAGAGGAGACACTCTATCGCCGTCAGC
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ACCATAGACCCCCCCGAGAGCCAAGGTCCTCGGACCCCGCCCAGCCCTGGTATCTGC
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GACAGCATGGCTGCCTCGCCCTCCCCAAAGAAAGATGTGCTGAGTCTCTCCGGTTTA
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TCTCCACTGGGTGCCAAGTCCTAGCTCCTCCTCCTGGGCTATATTCCTGACAAAAGT
TCCATATAGACACCAAGGAGGCGGAGGCGCTCCTCCCTGCCTCAGTGGCTCTGGGTA
CCTGCAAGCAGAACTTCCAAAGAGAGTTAAAAGCAGCAGCCCCGGCAACTCTGGCTC
CAGGCAGAAGGAGAGGCCCGGTGCAGCTGAGGTTCCCGACACCAGAAGCTGTTGGGA
GAAAGCAATACGTTTGTGCAGAATCTCTATGTATATTCTATTTTATTAAATTAATTG
AATCTAGTATATGCGGGATGTACGACATTTTGTGACTGAAGAGACTTGTTTCCTTCT
ACTTTTATGTGTCTCAGAATATTTTTGA

FIG. 3

SEQ.ID.NO.5. Coding sequence for human calcium channel alpha1G-c (2273 amino acids)

MDEEEDGAGAEESGQPRSFMRLNDLSGAGGRPGPGSAEKDPGSADSEAEGLPYPALAP
VVFFYLSQDSRPRSWCLRTVCNPWFERISMLVILLNCVTLGMFRPCEDIACDSQRCRI
LQAFDDFIFAFFAVEMVVKMVALGIFGKKCYLGDTWNRLDFFIVIAGMLEYSLDLQNV
SFSAVRTVRVLRPLRAINRVPSMRILVTLLLDTLPMLGNVLLLCFFVFFIFGIVGVQL
WAGLLRNRCFLPENFSLPLSVDLERYYQTENEDESPFICSQPRENGMRSCRSVPTLRG
DGGGGPPCGLDYEAYNSSSNTTCVNWNQYYTNCSAGEHNPFKGAINFDNIGYAWIAIF
QVITLEGWVDIMYFVMDAHSFYNFIYFILLIIVGSFFMINLCLVVIATQFSETKQRES
QLMREQRVRFLSNASTLASFSEPGSCYEELLKYLVYILRKAARRLAQVSRAAGVRVGL
LSSPAPLGGQETQPSSSCSRSHRRLSVHHLVHHHHHHHHHYHLGNGTLRAPRASPEIQ
DRDANGSRRLMLPPPSTPALSGAPPGGAESVHSFYHADCHLEPVRCQAPPPRSPSEAS
GRTVGSGKVYPTVHTSPPPETLKEKALVEVAASSGPPTLTSLNIPPGPYSSMHKLLET
QSTGACQSSCKISSPCLKADSGACGPDSCPYCARAGAGEVELADREMPDSDSEAVYEF
TQDAQHSDLRDPHSRRQRSLGPDAEPSSVLAFWRLICDTFRKIVDSKYFGRGIMIAIL
VNTLSMGIEYHEQPEELTNALEISNIVFTSLFALEMLLKLLVYGPFGYIKNPYNIFDG
VIVVISVWEIVGQQGGGLSVLRTFRLMRVLKLVRFLPALQRQLVVLMKTMDNVATFCM
LLMLFIFIFSILGMHLFGCKFASERDGDTLPDRKNFDSLLWAIVTVFQILTQEDWNKV
LYNGMASTSSWAALYFIALMTFGNYVLFNLLVAILVEGFQAEEISKREDASGQLSCIQ
LPVDSQGGDANKSESEPDFFSPSLDGDGDRKKCLALVSLGEHPELRKSLLPPLIIHTA
ATPMSLPKSTSTGLGEALGPASRRTSSSGSAEPGAAHEMKSPPSARSSPHSPWSAASS
WTSRRSSRNSLGRAPSLKRRSPSGERRSLLSGEGQQSQDQEESSEEERASPAGSDHRH
RGSLEREAKSSFDLPDTLQVPGLHRTASGRGSASEHQDCNGKSASGRLARALRPDDPP
LDGDDADDEGNLSKGERVRAWIRARLPACCLERDSWSAYIFPPQSRFRLLCHRIITHK
MFDHVVLVIIFLNCITIAMERPKIDPHSAERIFLTLSNYIFTAVFLAEMTVKVVALGW
CFGEQAYLRSSWNVLDGLLVLISVIDILVSMVSDSGTKILGMLRVLRLLRTLRPLRVI
SRAQGLKLVVETLMSSLKPIGNIVVICCAFFIIFGILGVQLFKGKFFVCQGEDTRNIT
NKSDCAEASYRWVRHKYNFDNLGQALMSLFVLASKDGWVDIMYDGLDAVGVDQQPIMN
HNPWMLLYFISFLLIVAFFVLNMFVGVVVENFHKCRQHQEEEEARRREEKRLRRLEKK
RRSKEKQMAEAQCKPYYSDYSRFRLLVHHLCTSHYLDLFITGVIGLNVVTMAMEHYQQ
PQILDEALKICNYIFTVIFVLESVFKLVAFGFRRFFQDRWNQLDLAIVLLSIMGIPLE
QIEVNASLPINPTIIRIMRVLRIARVLKLLKMAVGMRALLDTVMQALPQVGNLGLLFM
LLFFIFAALGVELFGDLECDETHPCEGLGRHATFRNFGMAFLTLFRVSTGDNWNGIMK
DTLRDCDQESTCYNTVISPIYFVSFVLTAQFVLVNVVIAVLMKHLEESNKEAKEEAEL
EAELELEMKTLSPQPHSPLGSPFLWPGVEGPDSPDSPKPGALHPAAHARSASHFSLEH
PTMQPHPTELPGPDLLTVRKSGVSRTHSLPNDSYMCRHGSTAEGPLGHRGWGLPKAQS
GSVLSVHSQPADTSYILQLPKDAPHLLQPHSAPTWGTIPKLPPPGRSPLAQRPLRRQA
AIRTDSLDVQGLGSREDLLAEVSGPSPPLARAYSFWGQSSTQAQQHSRSHSKISKHMT
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AQSCQRRPTSWLDEQRRHSIAVSCLDSGSQPHLGTDPSNLGGQPLGGPGSRPKKKLSP
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GLSSDPADLDP

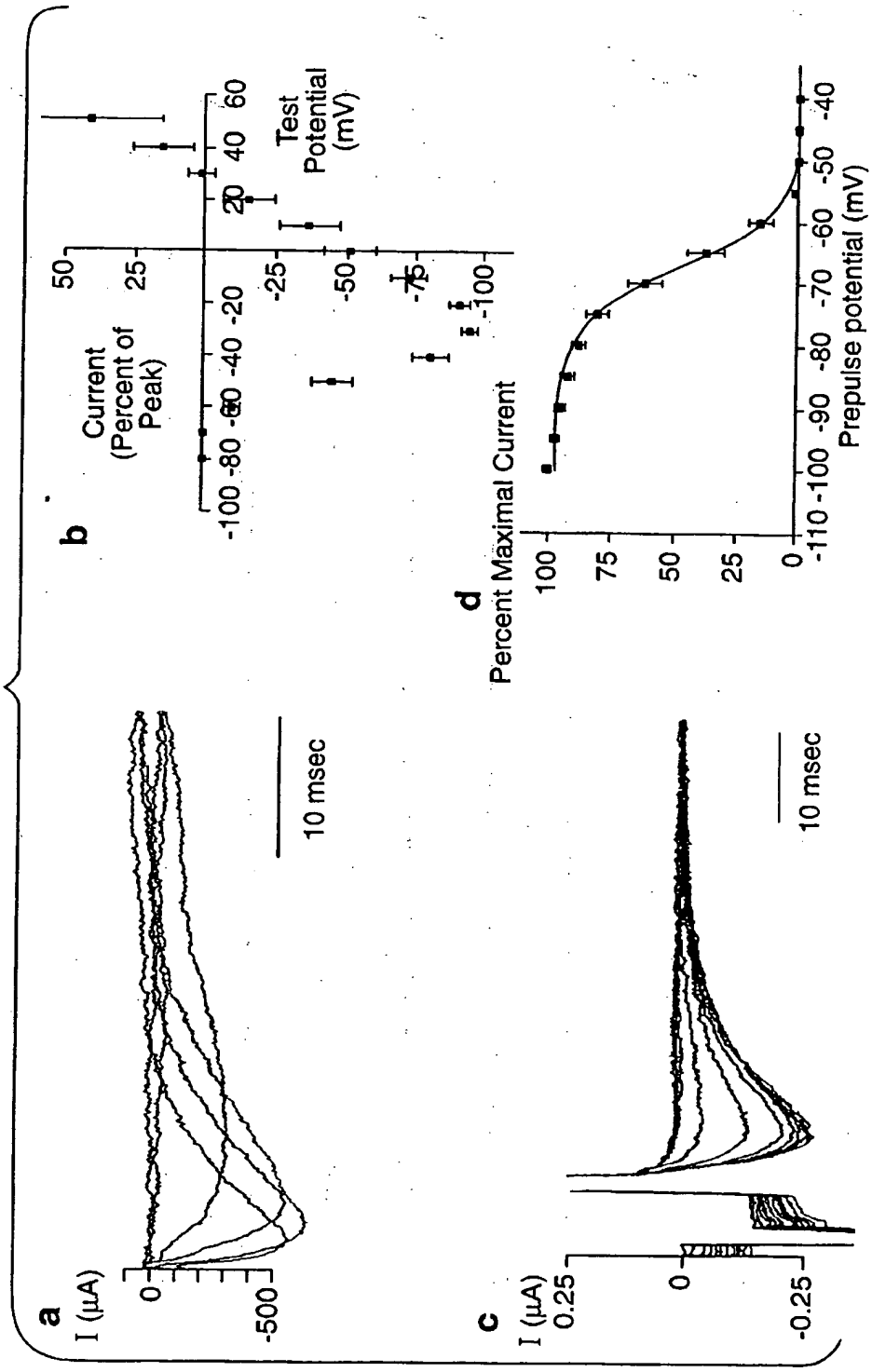
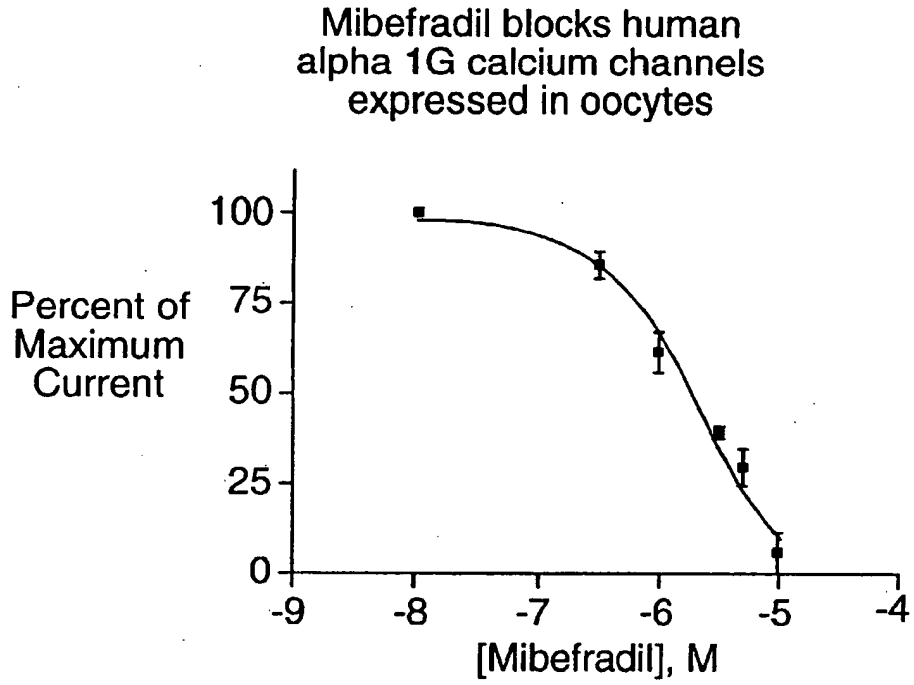

a
I (μA)
0
-500
10 msec
b
Current (Percent of Peak)
50
25
-25
-50
-75
-100
-100 -80 -60 -40 -20 20 40 60
Test Potential (mV)
c
I (μA)
0.25
0
-0.25
10 msec
d
Percent Maximal Current
100
75
50
25
0
-110 -100 -90 -80 -70 -60 -50 -40
Prepulse potential (mV)

FIG. 4

FIG. 5


Mibefradil blocks human
alpha 1G calcium channels
expressed in oocytes
Percent of
Maximum
Current
100
75
50
25
0
-9
-8
-7
-6
-5
-4
[Mibefradil], M

DNA ENCODING HUMAN ALPHA1G-C T-TYPE CALCIUM CHANNEL

BACKGROUND OF THE INVENTION

Voltage activated calcium channels play important roles including neuroexcitation, neurotransmitter and hormone secretion, and regulation of gene transcription through Ca-dependent transcription factors. Their functions depend in part on their cellular localization and their gating properties (characteristics of their opening, inactivation, deactivation, and recovery from inactivation). Five general classes of voltage activated calcium channels have been observed in various neuronal and non-neuronal tissues. The complement of channel subunits and the subcellular localization of the expressed voltage activated calcium channels determine the functional cellular properties.

Diversity of Voltage-gated Ca Channels Fall into Two Major Categories: Low Voltage Activated (LVA) and High Voltage Activated (HVA)

A conserved general structure for all cloned voltage-gated calcium channel alpha subunits (the pore-forming subunit) has been identified. It consists of 4 domains with homology to the domains present in voltage-gated K and Na channels. Each domain contains 6 membrane spanning regions (S1–S6) and a pore region (P) located between S5 and S6. The extracellular loops are generally very short; intracellular loops contain sites that are modulated by phosphorylation and can interact with other effectors. However, there are notable differences in the lengths of the S5–S6 loop of domain I and the intracellular loop between domains I and II among alpha subunits.

Different calcium channels are best distinguished by their pharmacological profiles since their electrophysiological properties differ depending on the cell type or tissue in which they are expressed, presumably because of modulation by cellular proteins, for instance kinases, and also auxiliary calcium channel subunits.

The HVA channel classes are thought to be composed of at least 3 or 4 different subunits: α1 (which contains the pore), beta (β) and α2δ. In skeletal muscle a γ subunit also co-precipitates with the skeletal channel complex. Recently two gamma-like subunits have been cloned from brain—one of which is the gene mutated in the stargazer mutant mouse (Black et al., 1999; Letts et al., 1998). The subunit composition has been proved for only the skeletal L-type (α1 α2δ β γ) and brain N-type (α1 α2δ β) channels (Perez-Reyes and Schneider, 1995). These channels generally require large membrane depolarizations for activation (~30 mV from the resting potential (RP)). Four classes of HVA calcium channels have been identified on the basis of electrophysiological, pharmacological and molecular data. These classes include L-type (encoded by at least 4 genes (including a α1 subunits α1S (skeletal muscle), α1C, α1D (neuroendocrine), and α1F (retinal)), N-type (α1B; (Williams et al., 1992)), P/Q-type (α1A) and R-type (encoded by at least the α1E gene).

HVA α1 families are strongly affected by co-expression of the cytoplasmically localized β subunit, particularly the expression levels of functional cell surface channels and the electrophysiological response of the channel (ie., kinetics). β subunits interact with a specific sequence in the I-II intracellular loop to increase the number of functional channels and alter the activation and inactivation properties of the channel complex (Furukawa et al., 1998). There are at least 4β genes that are alternatively spliced (β1a-c; β2a-c; β3; β4;(Perez-Reyes and Schneider, 1995)); the effect of each of these βs on α1 function appears to depend on the α1

class. Interestingly, mutants in β (Cchβ4) produce ataxia and seizures in the lethargic (lh) mouse (Burgess et al., 1997). α2δ subunits also modulate α1 function and the known gene co-segregates with malignant hyperthermia phenotype in certain families (Iles et al., 1994).

The physiological roles of HVA channels depend on subcellular location of the channel and tissue type. Subcellular location varies among tissues but have been shown to be important in neurotransmitter and hormone release, action potential duration, excitation-contraction coupling in muscle cells, and gene expression (Miller, 1987).

There are at least three genes in the T-type family of LVA calcium channels (α1G, α1H, and α1I) (Perez-Reyes, 1998). Their structure differs from that of the HVA channels in a number of important ways. The I-II intracellular linker is much longer (~400 amino acids) than that of the known HVA channels. The Domain I S5-P extracellular linker is longer than that of the HVA channels and may be a good target for drug interactions with this channel. β does not appear to be associated with α1 in this class and they lack the canonical sequence that is known to be crucial for beta subunit binding (Lambert et al., 1997; Leuranguer et al., 1998). Anti-sense experiments directed against all known beta's show a decrease in the expression of HVA calcium channels but not LVA calcium channels in nodose ganglion neurons (Lambert et al., 1997).

Other proteins or cellular environments may be required for robust T-channel expression since α1G expressed in oocytes or HEK293 cells produces dramatically different current magnitudes in these two cell types (Perez-Reyes, 1998).

T-type calcium currents have been observed in vivo in many cell types in the peripheral and central nervous systems including thalamus, inferior olive, cerebellar Purkinje cells, lateral habenular cells, dorsal horn neurons, sensory neurons (DRG, nodose), cholinergic forebrain neurons, hippocampal intemeurons, CA1, CA3 dentate gyrus pyramidal cells, basal forebrain neurons, amygdaloid neurons (Talley et al., 1999). T-type channels are prominent in the soma and dendrites of neurons that reveal robust Ca-dependent burst firing behaviors such as the thalamic relay neurons and cerebellar Purkinje cells (Huguenard, 1996).

Physiological Roles and Therapeutic Areas

T-type calcium channels are involved in the generation of low threshold spikes to produce burst firing (Huguenard, 1996). These channels differ from HVA channels in that they have some probability of opening at the resting membrane potential. Because their steady state inactivation curve is shifted toward negative voltages compared to HVA channels (ie., half the channels are not inactivated and are able to be opened by a depolarizing voltage step at voltages more negative than the resting membrane potential (RP)), there is a window current near the RP (ie., a portion of the T-channels are open at RP). Low threshold spikes and rebound burst firing is prominent in neurons from inferior olive, thalamus, hippocampus and neocortex (Huguenard, 1996).

T-type channels promote oscillatory behavior which has important consequences for epilepsy. The ability of a cell to fire low threshold spikes is critical in the genesis of oscillatory behavior and increased burst firing (groups of action potentials separated by about 50–100 ms). T-type calcium channels are thought to play a significant role in absence epilepsy, a type of generalized non-convulsive seizure. The evidence that voltage-gated calcium currents contribute to the epileptogenic discharge, including seizure maintenance and propagation includes 1) a specific enhancement of

T-type currents in the reticular thalamic (nRT) neurons which are hypothesized to be involved in the genesis of epileptic seizures in a rat genetic model (GAERS) for absence epilepsy (Tsakiridou et al., 1995); 2) antiepileptics against absence petit mal epilepsy (ethosuximide and dimethadione) have been shown at physiologically relevant doses to partially depress T-type currents in thalamic (ventrobasal complex) neurons (Coulter et al., 1989; Kostyuk et al., 1992); and 3) T-type calcium channels underlie the intrinsic bursting properties of particular neurons that are hypothesized to be involved in epilepsy (nRT, thalamic relay and hippocampal pyramidal cells) (Huguenard, 1996). The rat α1G is highly expressed in thalamocortical relay cells (TCs) which are capable of generating prominent Ca^{2+}-dependent low-threshold spikes (Talley et al., 1999).

T-type channels play a critical role in thalamic oscillations and cortical synchrony, and their involvement has been directly implicated in the generation of cortical spike waves that are thought to underlie absence epilepsy and the onset of sleep (McCormick and Bal, 1997). Oscillations of neural networks are critical in normal brain function such during sleep-wave cycles. It is widely recognized that the thalamus is intimately involved in cortical rhythmogenesis. Thalamic neurons most frequently exhibit tonic firing (regularly spaced spontaneous firing) in awake animals, whereas phasic burst firing is typical of slow-wave sleep and may account for the accompanying spindling in the cortical EEG. The shift to burst firing occurs as a result of activation of a low threshold Ca^{2+} spike which is stimulated by synaptically mediated inhibition (ie., activated upon hyperpolarization of the RP). The reciprocal connections between pyramidal neurons in deeper layers of the neocortex, cortical relay neurons in the thalamus, and their respective inhibitory interneurons are believed to form the elementary pacemaking circuit.

T-type channels contribute to synaptic potentiation at the postsynaptic level since small changes in membrane potential (Vm) (either depolarizations (epsps; excitatory postsynaptic potentials) or hyperpolarizations (ipsps (inhibitory postsynaptic potentials); anode break exhaltation or rebound burst firing) can open T-type calcium channels. At the hyperpolarized Vm during the ipsp more T-type channels become available to open (they have recovered from inactivation) so that upon repolarization to the RP, a larger proportion of T channels are opened and this produces anode break exhaltation, a robust rebound burst firing as the low threshold Ca spike reaches threshold for Na channel activation and action potential generation. A burst of action potentials ride on top of the Ca-dependent depolarization. This phenomenon is particularly prominent in reticular thalamic neurons (Huguenard, 1996).

T-type channels can be involved in transmitter release. In cells where T-channels are located at the presynaptic terminal, they promote neurotransmitter release (Ahnert-Hilger et al., 1996; Arnoult et al., 1997)

T-type channels contribute to spontaneous fluctuations in intracellular Ca concentrations $[Ca]_i$. They are important in pacemaker activity and therefore heart rate in the heart, and in vesicle release from non-excitable cells (Ertel et al., 1997).

T-type calcium currents are expressed differentially in different subpopulations of adult rat dorsal root ganglion (DRG) neurons. T-type currents were present at moderate densities in small diameter Type 1 and 3 cells, the former having TTX-resistant Na currents, long duration action potentials and capsaicin sensitivity (consistent with a C type nociceptive neuron) and the latter having short action potential durations, no capsaicin sensitivity (consistent with a Aδ nociceptive or Aα/β neurons) (Cardenas et al., 1995). There appear to be different types of LVA currents expressed in adult rat sensory neurons based on differential sensitivity to nM concentrations of nimodipine (Formenti et al., 1993). Because of the role of the T type calcium channel in contributing to near threshold membrane excitability, selective suppression of the T channels will decrease neuronal hyperexcitability (painful neuropathies) and raise the threshold for the perception of pain (central pain syndromes).

A specific blocker for T-type calcium channels in the pacemaker cells and conduction fibers in the heart might demonstrate "pure" bradycardic (slowing the heart rate) properties since T channels are not usually present in the ventricular myocytes of man. Drugs that block the T-type channel in specific conformational states might allow treatment of tachycardia (by decreasing the heart rate) while having little effect on the inotropic properties of the normal heart (Rousseau et al., 1996). A cardiomyopathic disease (genetic Syrian hamster model) is a result of Ca-overload due to an increased expression of T-type calcium channels in ventricular myocytes (Sen and Smith, 1994). There are increased T-type currents in atrial myocytes from adult rats with growth hormone-secreting tumors (Xu and Best, 1990). A specific T-type calcium channel blocker would act as a cardioprotectant in these cases.

T-type channels in adrenal zona fasciculata cells of the adrenal cortex have been shown to modulate cortisol secretion (Enyeart et al., 1993). Cortisol is the precursor for glucocorticoids and prolonged exposure to glucocorticoids causes breakdown of peripheral tissue protein, increased glucose production by the liver and mobilization of lipid from the fat depots. Furthermore, individuals suffering from anxiety and stress produce too high levels of glucocorticoids and drugs that would regulate these levels are sought after (eg., antagonists to CRF).

T-type calcium channels may be involved in release of nutrients from testis Sertoli cells. T-type calcium channels are expressed on immature rat Sertoli cells (Lalevee et al., 1997). Sertoli cells are testicular cells that are thought to play a major role in sperm production. The intimate juxtaposition of the developing germ cells with the Sertoli cells suggests the latter pay a role in supporting and nurturing the gametes. Sertoli cells secrete a number of proteins including transport proteins, hormones and growth factors, enzymes which regulate germinal cell development and other biological processes related to reproduction (Griswold, 1988). They secrete the peptide hormone inhibin B, an important negative feedback signal to the anterior pituitary. They assist in spermiation (the final detachment of the mature spermatozoa from the Sertoli cell into the lumen) by releasing plasminogen activator which produces proteolytic enzymes. While the role of T channels in not known, they may be important in the release of nutrients, inhibin B, and/or plasminogen activator.

Inhibition of T-type calcium channels in sperm during gamete interaction inhibits zona pellucida-dependent Ca^{2+} elevations and inhibits acrosome reactions, thus directly linking sperm T-type calcium channels to fertilization (Arnoult et al., 1996).

T-type calcium channels have also been implicated in cellular growth and proliferation, particularly in the cardiovascular system (Katz, 1999; Lijnen and Petrov, 1999; Richard and Nargeot, 1998; Wang et al., 1993).

Tremor can be controlled through the basal ganglia and the thalamus, regions in which T type calcium channels are

strongly expressed (Talley et al., 1999). T-type calcium channels have been implicated in the pathophysiology of tremor since the anti-epileptic drug ethosuximide is used for treating tremor, in particular, tremor associated with Parkinson's disease, essential tremor, or cerebellar disease (U.S. Pat. No. 4,981,867; D. A. Prince).

Pharmacology

There are no known specific blockers of the T-type class of calcium channel. There are ions (ex. Ni^{+2}) that are more effective toward blocking T-type calcium channels vs. HVA channels, and there are a few drugs that block T channels with higher affinity than HVA channels. A number of pharmacological blockers have differential effects on T type calcium currents expressed in different cell types (see Table 1 from (Todorovic and Lingle, 1998)), however there is a diversity of pharmacological profiles of T-type currents. The differential sensitivity of the currents to antagonists may be due to different subunit structure (Perez-Reyes, 1998) as well as cellular environments. T-type calcium channel alpha subunit genes, like the genes for HVA channels, reveal alternative splicing (Lee et al., 1999 Biophys J 76:A408). Extracellular and intracellular loops of individual T-type calcium channel clones show marked diversity amongst themselves and even less homology to HVA channels.

Mibefradil ((1S,2S)-2-[2-[[3-(1H-benzimidazol-2-yl) propyl]methyl-amino]ethyl]-6-fluoro-1-isopropyl-1,2,3,4-tetrahydronaphthalen-2-yl methoxyacetate) blocks the T-type calcium channel by preferentially intereacting with inactivated state. Thus, in a cell type with a relatively low RP (—50 mV) such as the smooth muscle cells, nearly all T channels will be blocked by mibefradil, whereas in cells with a very negative RP such as cardiac myocytes most of the T channels are not inactivated and therefore will not be blocked by mibefradil (Bezprozvanny and Tsien, 1995). Mibefradil had a complex blocking action on the mouse alpha1G when applied from holding potentials of −60 and could best be fit by fitting to 2 populations of sites (Klugbauer et al., 1999). The high affinity component was reduced at −100 mV. The most prominent (low affinity) site had an IC_{50} value for mibefradil of ~400 nM.

Ethosuximide is used to treat absence epilepsy and at therapeutically relevant concentrations (0.25–0.75 mM) (Sherwin, 1989) partially blocks T-type currents in some preparations (Coulter et al., 1989). Ethosuximide has different affinities for T-type calcium channels in different tissues. The majority of T type currents from guinea pig or rat ventrobasal thalamic neurons revealed an IC_{50} for mibefradil of ~500 μM and a maximal block of ~40% block at 1 mM (Coulter et al., 1989). Interestingly, there was no effect of ethosuximide on T-currents in 25% of the TCs tested (Coulter et al., 1989). In hippocampal CA3 neurons, all components of the LVCC were insensitive to ethosuximide at 250 μM or 1 mM. If T-type calcium channels underlie the LVCC in these cells, then the drug had no effect on these T-type calcium channels (Avery and Johnston, 1996). The T-type calcium channels from dorsal root ganglion neurons from one-day-old rats have higher affinity for ethosuximide than thalamic neurons (Kd for T-current is 7 μM vs 15 μM for L-type current) with a maximal block of 100% (Kostyuk et al., 1992). The human alpha1H is insensitive to ethosuximide (Williams et al., WO 9928342; Williams et al., 1999).

Ni2+ is thought to act not only at the pore region but also at another unknown location on the channel protein (Zamponi et al., 1996). The mouse alpha1G has a very low sensitivity to Ni^{2+} as opposed to other T-type channels (Klugbauer et al., 1999). The human alpha1H expressed in oocytes has an IC50 for Ni^{2+} of about 6 μM (Williams et al., WO 9928342).

Amiloride, an antagonist at numerous receptors, channels and exchangers, is a low affinity antagonist at T-type calcium channels. There are noted differences in sensitivity of T currents to amiloride (Todorovic and Lingle, 1998). The effects of amiloide are highly variable depending on the cell type, with EC50's ranging from 50 to >1000 μM, suggesting that different levels of T-type channel expression in different cells or different channel complexes within different cells (Huguenard, 1996). For instance, the human alpha1H expressed in oocytes has an IC50 for amiloride of about 20 μM (Williams et al., WO 9928342).

NPPB (5-Nitro-2-(3-phenylpropylamino)benzoic acid) has been used to isolate N-type calcium channels (Stea et al., 1999) and was used in studies on the present invention to isolate T-type calcium channels. However, we found NPPB blocked halpha1G-c currents. NPPB has been shown to block voltage-sensitive calcium currents (Kirkup et al., 1996), and, more specifically, L-type calcium currents (Doughty et al., 1998). Interestingly, NPPB reduced the Ca^{2+} resting current and altered the spike frequency of isolated cockroach dorsal unpaired median neurons (Heine and Wicher, 1998). The resting calcium current may be mediated by a T-type calcium channel, but this has yet to be confirmed.

SUMMARY OF THE INVENTION

A DNA molecule encoding a novel isoform of the human T-type low voltage activated calcium channel (alpha1G-c) has been cloned and characterized. The biological and structural properties of this protein is disclosed, as is the amino acid and nucleotide sequence. The recombinant protein is useful to identify modulators of the alpha1G-c calcium channel. Modulators identified in the assays disclosed herein are useful as therapeutic agents and are candidates for the treatment disorders that are mediated by human alpha1G-c activity. Such activities that may be mediated by human alpha1G-c include, epilepsy, schizophrenia, depression, sleep disorders, stress, endocrine disorders, respiratory disorder, peripheral muscle disorders, muscle excitability, Cushing's disease, fertilization, contraception, disorders involving neuronal firing regulation, respiratory disorders, hypertension, cardiac rhythm, potentiation of synaptic signals, improving arterial compliance in systolic hypertension, vascular tone such as by decreasing vascular swelling, cellular growth (protein synthesis, cell differentiation, and proliferation), cardiac hypertrophy, cardiac fibrosis, atherosclerosis, cardiovascular disorders, including but not limited to: myocardial infarct, cardiac arrhythmia, heart failure and angina pectoris. The recombinant DNA molecules, and portions thereof, are useful for isolating homologues of the DNA molecules, identifying and isolating genomic equivalents of the DNA molecules, and identifying, detecting or isolating mutant forms of the DNA molecules.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1, Panel A, Panel B and Panel C—The nucleotide sequence of coding region of human calcium channel alpha1G-c is shown (6822 bp including the stop codon).

FIG. 2, Panel A, Panel B and Panel C—The nucleotide sequence of human calcium channel alpha1G-c is shown including 511 bp 5' UT and 397 bp 3'UT.

FIG. 3—The amino acid sequence of human calcium channel alpha1G-c is shown (2273 amino acids).

FIG. 4—Functional expression of human calcium channel alpha1G-c in Xenopus oocytes is shown: activation by

depolarizing voltage steps (a,b) and steady state inactivation (c,d). a) An oocyte bathed in 40 mM $BaCl_2$ saline was challenged with a depolarizing voltage protocol from a holding potential of −100 mV. 40 msec test pulses were applied from −70 to −20 mV in increments of 10 mV. b) The current-voltage relationship obtained from 9 oocytes bathed in ND96. Currents activated near −60 mV and reversed sign near +30 mV. Peak currents were elicited by steps to about −30 mV. c) The voltage-dependence of inactivation of an oocyte bathed in 40 mM $BaCl_2$ was determined using a standard voltage protocol. Four sec voltage steps to −100 to −45 mV (in increments of 5 mV) were followed by a 5 msec step to −100 mV, followed by a step to −30 mV. The currents elicited at −30 mV are shown after the positive-going capacitative transient. The prepulse voltage that inactivated half the channels ($V_{0.5}$) was about −70 mV. d) The voltage dependence of inactivation is shown for oocytes bathed in ND96 (n=9 experiments).

FIG. 5—Pharmacological characterization of human alpha1G-c expressed in Xenopus oocytes: dose dependent block by mibefradil. The responses to the indicated concentrations of mibefradil were bath applied to oocytes expressing human calcium channel alpha1G-c cRNA. Shown are 1–3 concentrations tested on 7 individual oocytes. The IC50 was 2.5 μM with a 95% confidence interval of 1.3 to 4.9 μM. Oocytes were bathed in ND96.

DETAILED DESCRIPTION

The present invention relates to DNA encoding human calcium channel alpha1G-c that was isolated from a human thalamus cDNA library. Human calcium channel alpha1G-c, as used herein, refers to protein that can specifically function as a low voltage activated calcium channel.

The sequence presented in this invention is a homolog of the rat alpha1G accession # AF027984 (Perez-Reyes et al., 1998), and is similar to the human alpha1G "a" isoform (accession # AF126966) with the exception that the sequence presented herein contains a 23 amino acid insert in the second intracellular loop between domains I and II that is missing in both sequences. The 23 amino acid insert contains a putative CKII phosphorylation site at S971. This 23 amino acid insert is 91 and 87% identical to homologous sequences in rat (AF125161) and mouse (AJ012569), respectively, two proteins otherwise dissimilar to human alphaG-c since they contain an insert at alpha1G amino acid 1575. The putative casein kinase II phosphorylation site in the human alphaG-c insert is not conserved in the equivalent rat or mouse sequences. The previously described human full length cDNA (AF126966) produces functional channels (Monteil, a et al., 1999 Cloning and molecular characterization of a1G and a1I isoforms of human T-type Ca2+ channels. Biophys. Abst: A408) but a complete description of its functional and structural characteristics has not been reported. The present invention is thus the first report, to our knowledge, of a detailed characterization of the human alpha1G-c T-type calcium channel. There are 2 partial human sequences that are identical to regions of the present invention submitted by E. Perez-Reyes (AF029229; AF029228). AF029228 begins at alpha1G-c at amino acid 1186 and ends at amino acid 1504; AF029229 begins at amino acid 1827 and ends at the TGA stop codon.

The complete amino acid sequence of human calcium channel alpha1G has been previously described, however, the present invention is a novel isoform that was not previously known. This is the first reported cloning of a full length DNA molecule encoding the "c" isoform of the human calcium channel alpha1G. It is predicted that a wide variety of cells and cell types will contain the described channel.

Other cells and cell lines may also be suitable for use to isolate human calcium channel alpha1G-c. Selection of suitable cells may be done by screening for human calcium channel alpha1G-c activity in whole cells or cell extracts. Human calcium channel alpha1G-c activity can be monitored by direct measurement of a low depolarizing voltage-induced Ca^{2+} influx or Ca currents through the human calcium channel alpha1G-c. Cells that possess human calcium channel alpha1G-c activity in this assay may be suitable for the isolation of human calcium channel alpha1G-c DNA or mRNA.

Any of a variety of procedures known in the art may be used to molecularly clone human calcium channel alpha1G-c. These methods include, but are not limited to, direct functional expression of the human calcium channel alpha1G-c genes following the construction of a human calcium channel alpha1G-c -containing cDNA library in an appropriate expression vector system. Another method is to screen human calcium channel alpha1G-c -containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a labelled oligonucleotide probe designed from the amino acid sequence of the human calcium channel alpha1G-c insert. An additional method consists of screening a human calcium channel alpha1G-c-containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a partial cDNA encoding the human calcium channel alpha1G-c protein. This partial cDNA is obtained by the specific PCR amplification of human calcium channel alpha1G-c DNA fragments through the design of degenerate oligonucleotide primers from the amino acid sequence of the purified human calcium channel alpha1G-c protein.

Another method is to isolate RNA from human calcium channel alpha1G-c-producing cells and translate the RNA into protein via an in vitro or an in vivo translation system. The translation of the RNA into a peptide a protein will result in the production of at least a portion of the human calcium channel alpha1G-c protein which an be identified by, for example, immunological reactivity with an anti-human calcium channel alpha1G-c antibody or by biological activity of human calcium channel alpha1G-c protein. In this method, pools of RNA isolated from human calcium channel alpha1G-c-producing cells can be analyzed for the presence of an RNA that encodes at least a portion of the human calcium channel alpha1G-c protein. Further fractionation of the RNA pool can be done to purify the human calcium channel alpha1G-c RNA from non-human calcium channel alpha1G-c RNA. The peptide or protein produced by this method may be analyzed to provide amino acid sequences, which in turn are used to provide primers for production of human calcium channel alpha1G-c cDNA, or the RNA used for translation can be analyzed to provide nucleotide sequences encoding human calcium channel alpha1G-c and produce probes for this production of human calcium channel alpha1G-c cDNA. This method is known in the art and can be found in, for example, Maniatis, T., Fritsch, E. F., Sambrook, J. in Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 1989.

It is readily apparent to those skilled in the art that other types of libraries, as well as libraries constructed from other cells or cell types, may be useful for isolating human calcium channel alpha1G-c-encoding DNA. Other types of libraries include, but are not limited to, cDNA libraries derived from other cells and genomic DNA libraries that include YAC (yeast artificial chromosome) and cosmid libraries.

It is readily apparent to those skilled in the art that suitable cDNA libraries may be prepared from cells or cell lines which have human calcium channel alpha1G-c activity. The selection of cells or cell lines for use in preparing a cDNA library to isolate human calcium channel alpha1G-c cDNA may be done by first measuring cell associated human calcium channel alpha1G-c activity using the measurement of calcium regulated biological activity.

Preparation of cDNA libraries can be performed by standard techniques well known in the art. Well known cDNA library construction techniques can be found for example, in Maniatis, T., Fritsch, E. F., Sambrook, J., Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor A Laboratory, Cold Spring Harbor, N.Y., 1989).

It is also readily apparent to those skilled in the art that DNA encoding human calcium channel alpha1G-c may also be isolated from a suitable genomic DNA library. Construction of genomic DNA libraries can be performed by standard techniques well known in the art. Well known genomic DNA library construction techniques can be found in Maniatis, T., Fritsch, E. F., Sambrook, J. in Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989).

In order to clone the human calcium channel alpha1G-c gene by the above methods, the amino acid sequence of human calcium channel alpha1G-c may be necessary. To accomplish this, human calcium channel alpha1G-c protein may be purified and partial amino acid sequence determined by automated sequencers. It is not necessary to determine the entire amino acid sequence, but the linear sequence of two regions of 6 to 8 amino acids from the protein is determined for the production of primers for PCR amplification of a partial human calcium channel alpha1G-c DNA fragment.

Once suitable amino acid sequences have been identified, the DNA sequences capable of encoding them are synthesized. Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and therefore, the amino acid sequence can be encoded by any of a set of similar DNA oligonucleotides. Only one member of the set will be identical to the human calcium channel alpha1G-c sequence but will be capable of hybridizing to human calcium channel alpha1G-c DNA even in the presence of DNA oligonucleotides with mismatches. The mismatched DNA oligonucleotides may still sufficiently hybridize to the human calcium channel alpha1G-c DNA to permit identification and isolation of human calcium channel alpha1G-c encoding DNA. DNA isolated by these methods can be used to screen DNA libraries from a variety of cell types, from invertebrate and vertebrate sources, and to isolate homologous genes.

Purified biologically active human calcium channel alpha1G-c may have several different physical forms. Human calcium channel alpha1G-c may exist as a full-length nascent or unprocessed polypeptide, or as partially processed polypeptides or combinations of processed polypeptides. The full-length nascent human calcium channel alpha1G-c polypeptide may be posttranslationally modified by specific proteolytic cleavage events, which result in the formation of fragments of the full-length nascent polypeptide. A fragment, or physical association of fragments may have the full biological activity associated with human calcium channel alpha1G-c, however, the degree of human calcium channel alpha1G-c activity may vary between individual human calcium channel alpha1G-c fragments and physically associated human calcium channel alpha1G-c polypeptide fragments.

The cloned human calcium channel alpha1G-c DNA obtained through the methods described herein may be recombinantly expressed by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, and transferred into prokaryotic or eukaryotic host cells to produce recombinant human calcium channel alpha1G-c protein. Techniques for such manipulations are fully described in Maniatis, T. et al., supra, and are well known in the art.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic genes in a variety of hosts such as bacteria including *E. coli*, blue-green algae, plant cells, insect cells, fungal cells including yeast cells, and animal cells.

Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast or bacteria-animal cells or bacteria-fungal cells or bacteria-invertebrate cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one that causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

A variety of mammalian expression vectors may be used to express recombinant human calcium channel alpha1G-c in mammalian cells. Commercially available mammalian expression vectors which may be suitable for recombinant human calcium channel alpha1G-c expression, include but are not limited to, pMAMneo (Clontech), pcDNA3 (Invitrogen), pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593) pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), and IZD35 (ATCC 37565).

A variety of bacterial expression vectors may be used to express recombinant human calcium channel alpha1G-c in bacterial cells. Commercially available bacterial expression vectors which may be suitable for recombinant human calcium channel alpha1G-c expression include, but are not limited to pET vectors (Novagen) and pQE vectors (Qiagen).

A variety of fungal cell expression vectors may be used to express recombinant human calcium channel alpha1G-c in fungal cells such as yeast. Commercially available fungal cell expression vectors which may be suitable for recombinant human calcium channel alpha1G-c expression include but are not limited to pYES2 (InVitrogen) and Pichia expression vector (InVitrogen).

A variety of insect cell expression vectors may be used to express recombinant human calcium channel alpha1G-c in insect cells. Commercially available insect cell expression vectors that may be suitable for recombinant expression of human calcium channel alpha1G-c include but are not limited to pBlueBacII (InVitrogen).

DNA encoding human calcium channel alpha1G-c may be cloned into an expression vector for expression in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to bacteria such as

E. coli. fungal cells such as yeast, mammalian cells including but not limited to cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to drosophila and silkworm derived cell lines. Cell lines derived from mammalian species which may be suitable and which are commercially available, include but are not limited to, CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), L-cells, and HEK-293 (ATCC CRL1573).

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, protoplast fusion, lipofection, and electroporation. The expression vector-containing cells are clonally propagated and individually analyzed to determine whether they produce human calcium channel alpha1G-c protein. Identification of human calcium channel alpha1G-c expressing host cell clones may be done by several means, including but not limited to immunological reactivity with anti-human calcium channel alpha1G-c antibodies, and the presence of host cell-associated human calcium channel alpha1G-c activity.

Expression of human calcium channel alpha1G-c DNA may also be performed using in vitro produced synthetic mRNA. Synthetic mRNA or mRNA isolated from human calcium channel alpha1G-c producing cells can be efficiently translated in various cell-free systems, including but not limited to wheat germ extracts and reticulocyte extracts, as well as efficiently translated in cell based systems, including but not limited to microinjection into frog oocytes, with microinjection into frog oocytes being generally preferred.

To determine the human calcium channel alpha1G-c DNA sequence(s) that yields optimal levels of human calcium channel alpha1G-c activity and/or human calcium channel alpha1G-c protein, human calcium channel alpha1G-c DNA molecules including, but not limited to, the following can be constructed: the full-length open reading frame of the human calcium channel alpha1G-c cDNA encoding the approximately 252 kDa protein from approximately base 1 to approximately base 6822 (these numbers correspond to first nucleotide of first methionine and last nucleotide before the first stop codon) and several constructs containing portions of the cDNA encoding human calcium channel alpha1G-c protein. All constructs can be designed to contain none, all or portions of the 5' or the 3' untranslated region of human calcium channel alpha1G-c cDNA. Human calcium channel alpha1G-c activity and levels of protein expression can be determined following the introduction, both singly and in combination, of these constructs into appropriate host cells. Following determination of the human calcium channel alpha1G-c DNA cassette yielding optimal expression in transient assays, this human calcium channel alpha1G-c DNA construct is transferred to a variety of expression vectors, for expression in host cells including, but not limited to, mammalian cells, baculovirus-infected insect cells, *E. coli* and the yeast *S. cerevisiae.*

Host cell transfectants and microinjected oocytes may be used to assay both the levels of human calcium channel alpha1G-c channel activity and levels of human calcium channel alpha1G-c protein by the following methods. In the case of recombinant host cells, this involves the co-transfection of one or possibly two or more plasmids, containing the human calcium channel alpha1G-c DNA encoding one or more fragments or subunits. In the case of oocytes, this involves the co-injection of synthetic RNAs for human calcium channel alpha1G-c protein. Following an appropriate period of time to allow for expression, cellular protein is metabolically labelled with, for example ^{35}S-methionine for 24 hours, after which cell lysates and cell culture supernatants are harvested and subjected to immunoprecipitation with polyclonal antibodies directed against the human calcium channel alpha1G-c protein.

Other methods for detecting human calcium channel alpha1G-c activity involve the direct measurement of human calcium channel alpha1G-c activity in whole cells transfected with human calcium channel alpha1G-c cDNA or oocytes injected with human calcium channel alpha1G-c mRNA. Human calcium channel alpha1G-c activity is measured by biological characteristics of the host cells expressing human calcium channel alpha1G-c DNA. In the case of recombinant host cells expressing human calcium channel alpha1G-c patch voltage clamp techniques can be used to measure receptor activity and quantitate human calcium channel alpha1G-c protein. In the case of oocytes patch clamp as well as two-electrode voltage clamp techniques can be used to measure calcium channel alpha1G-c activity and quantitate human calcium channel alpha1G-c protein by determining single channel and whole cell conductances.

Levels of human calcium channel alpha1G-c protein in host cells are quantitated by immunoaffinity and/or ligand affinity techniques. Cells expressing human calcium channel alpha1G-c can be assayed for the number of human calcium channel alpha1G-c molecules expressed by measuring the amount of radioactive ligand binding to cell membranes. Human calcium channel alpha1G-c-specific affinity beads or human calcium channel alpha1G-c-specific antibodies are used to isolate for example ^{35}S-methionine labelled or unlabelled human calcium channel alpha1G-c protein. Labelled human calcium channel alpha1G-c protein is analyzed by SDS-PAGE. Unlabelled human calcium channel alpha1G-c protein is detected by Western blotting, ELISA or RIA assays employing human calcium channel alpha1G-c specific antibodies.

Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and therefore, the amino acid sequence can be encoded by any of a set of similar DNA oligonucleotides. Only one member of the set will be identical to the human calcium channel alpha1G-c sequence but will be capable of hybridizing to human calcium channel alpha1G-c DNA even in the presence of DNA oligonucleotides with mismatches under appropriate conditions. Under alternate conditions, the mismatched DNA oligonucleotides may still hybridize to the human calcium channel alpha1G-c DNA to permit identification and isolation of human calcium channel alpha1G-c encoding DNA.

DNA encoding human calcium channel alpha1G-c from a particular organism may be used to isolate and purify homologues of human calcium channel alpha1G-c from other organisms. To accomplish this, the first human calcium channel alpha1G-c DNA may be mixed with a sample containing DNA encoding homologues of human calcium channel alpha1G-c under appropriate hybridization conditions. The hybridized DNA complex may be isolated and the DNA encoding the homologous DNA may be purified therefrom.

It is known that there is a substantial amount of redundancy in the various codons that code for specific amino acids. Therefore, this invention is also directed to those DNA sequences that contain alternative codons that code for the eventual translation of the identical amino acid. For pur-

poses of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation. Also included within the scope of this invention are mutations either in the DNA sequence or the translated protein which do not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in functionality of the polypeptide. Such substitutions are well known and are described, for instance in *Molecular Biology of the Gene*, 4th Ed. Bengamin Cummings Pub. Co. by Watson et al.

It is known that DNA sequences coding for a peptide may be altered so as to code for a peptide having properties that are different than those of the naturally occurring peptide. Methods of altering the DNA sequences include, but are not limited to site directed mutagenesis. Examples of altered properties include but are not limited to changes in the affinity of an enzyme for a substrate or a receptor for a ligand.

As used herein, a "functional derivative" of human calcium channel alpha1G-c is a compound that possesses a biological activity (either functional or structural) that is substantially similar to the biological activity of human calcium channel alpha1G-c. The term "functional derivatives" is intended to include the "fragments," "variants," "degenerate variants," "analogs" and "homologues" or to "chemical derivatives" of human calcium channel alpha1G-c. The term "fragment" is meant to refer to any polypeptide subset of human calcium channel alpha1G-c. The term "variant" is meant to refer to a molecule substantially similar in structure and function to either the entire human calcium channel alpha1G-c molecule or to a fragment thereof. A molecule is "substantially similar" to human calcium channel alpha1G-c if both molecules have substantially similar structures or if both molecules possess similar biological activity. Therefore, if the two molecules possess substantially similar activity, they are considered to be variants even if the structure of one of the molecules is not found in the other or even if the two amino acid sequences are not identical. The term "analog" refers to a molecule substantially similar in function to either the entire human calcium channel alpha1G-c molecule or to a fragment thereof. The term "functional" with respect to a calcium channel activity means that the channel is able to provide for and regulate entry of calcium channel selective ions, including, but not limited to Ca+2 or Ba+2 or ions that block the flow of Ca+2 or Ba+2, in response to a stimulus and/or bind ligands with affinity for the channel. Preferably such channel activity is distinguishable, such as by electrophysiological, pharmacological and other means known to those of skill in the art, from any endogenous calcium channel activity that is in the host cell.

Following expression of human calcium channel alpha1G-c in a recombinant host cell, human calcium channel alpha1G-c protein may be recovered to provide human calcium channel alpha1G-c in active form. Several human calcium channel alpha1G-c purification procedures are available and suitable for use. As described above for purification of human calcium channel alpha1G-c from natural sources, recombinant human calcium channel alpha1G-c may be purified from cell lysates and extracts, or from conditioned culture medium, by various combinations of, or individual application of salt fractionation, ion exchange chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography and hydrophobic interaction chromatography.

In addition, recombinant human calcium channel alpha1G-c can be separated from other cellular proteins by use of an immunoaffinity column made with monoclonal or polyclonal antibodies specific for full length nascent human calcium channel alpha1G-c, polypeptide fragments of human calcium channel alpha1G-c or human calcium channel alpha1G-c subunits.

Monospecific antibodies to human calcium channel alpha1G-c are purified from mammalian antisera containing antibodies reactive against human calcium channel alpha1G-c or are prepared as monoclonal antibodies reactive with human calcium channel alpha1G-c using the technique of Kohler and Milstein, *Nature* 256: 495–497 (1975). Monospecific antibody as used herein is defined as a single antibody species or multiple antibody species with homogenous binding characteristics for human calcium channel alpha1G-c. Homogenous binding as used herein refers to the ability of the antibody species to bind to a specific antigen or epitope, such as those associated with the human calcium channel alpha1G-c, as described above. Human calcium channel alpha1G-c specific antibodies are raised by immunizing animals such as mice, rats, guinea pigs, rabbits, goats, horses and the like, with rabbits being preferred, with an appropriate concentration of human calcium channel alpha1G-c either with or without an immune adjuvant.

Preimmune serum is collected prior to the first immunization. Each animal receives between about 0.1 mg and about 1000 mg of human calcium channel alpha1G-c associated with an acceptable immune adjuvant. Such acceptable adjuvants include, but are not limited to, Freund's complete, Freund's incomplete, alum-precipitate, water in oil emulsion containing *Corynebacterium parvum* and tRNA. The initial immunization consists of human calcium channel alpha1G-c in, preferably, Freund's complete adjuvant at multiple sites either subcutaneously (SC), intraperitoneally (IP) or both. Each animal is bled at regular intervals, preferably weekly, to determine antibody titer. The animals may or may not receive booster injections following the initial immunization. Those animals receiving booster injections are generally given an equal amount of the antigen in Freund's incomplete adjuvant by the same route. Booster injections are given at about three-week intervals until maximal titers are obtained. At about 7 days after each booster immunization or about weekly after a single immunization, the animals are bled, the serum collected, and aliquots are stored at about −20° C.

Monoclonal antibodies (mAb) reactive with human calcium channel alpha1G-c are prepared by immunizing inbred mice, preferably Balb/c, with human calcium channel alpha1G-c. The mice are immunized by the IP or SC route with about 0.1 mg to about 10 mg, preferably about 1 mg, of human calcium channel alpha1G-c in about 0.5 ml buffer or saline incorporated in an equal volume of an acceptable adjuvant, as discussed above. Freund's complete adjuvant is preferred. The mice receive an initial immunization on day 0 and are rested for about 3 to about 30 weeks. Immunized mice are given one or more booster immunizations of about 0.1 to about 10 mg of human calcium channel alpha1G-c in a buffer solution such as phosphate buffered saline by the intravenous (IV) route. Lymphocytes, from antibody positive mice, preferably splenic lymphocytes, are obtained by removing spleens from immunized mice by standard procedures known in the art. Hybridoma cells are produced by mixing the splenic lymphocytes with an appropriate fusion partner, preferably myeloma cells, under conditions that will allow the formation of stable hybridomas. Fusion partners may include, but are not limited to: mouse myelomas P3/NS1/Ag4-1; MPC-11; S-194 and Sp 2/0, with Sp 2/0 being generally preferred. The antibody producing cells and

myeloma cells are fused in polyethylene glycol, about 1000 mol. wt., at concentrations from about 30% to about 50%. Fused hybridoma cells are selected by growth in hypoxanthine, thymidine and aminopterin supplemented Dulbecco's Modified Eagles Medium (DMEM) by procedures known in the art. Supernatant fluids are collected from growth positive wells on about days 14, 18, and 21 and are screened for antibody production by an immunoassay such as solid phase immunoradioassay (SPIRA) using human calcium channel alpha1G-c as the antigen. The culture fluids are also tested in the Ouchterlony precipitation assay to determine the isotype of the mAb. Hybridoma cells from antibody positive wells are cloned by a technique such as the soft agar technique of MacPherson, Soft Agar Techniques, in Tissue Culture Methods and Applications, Kruse and Paterson, Eds., Academic Press, 1973.

Monoclonal antibodies are produced in vivo by injection of pristane primed Balb/c mice, approximately 0.5 ml per mouse, with about 2×10^6 to about 6×10^6 hybridoma cells about 4 days after priming. Ascites fluid is collected at approximately 8–12 days after cell transfer and the monoclonal antibodies are purified by techniques known in the art.

In vitro production of anti-human calcium channel alpha1G-c mAb is carried out by growing the hybridoma in DMEM containing about 2% fetal calf serum to obtain sufficient quantities of the specific mAb. The mAb are purified by techniques known in the art.

Antibody titers of ascites or hybridoma culture fluids are determined by various serological or immunological assays which include, but are not limited to, precipitation, passive agglutination, enzyme-linked immunosorbent antibody (ELISA) technique and radioimmunoassay (RIA) techniques. Similar assays are used to detect the presence of human calcium channel alpha1G-c in body fluids or tissue and cell extracts.

It is readily apparent to those skilled in the art that the above described methods for producing monospecific antibodies may be utilized to produce antibodies specific for human calcium channel alpha1G-c polypeptide fragments, or full-length nascent human calcium channel alpha1G-c polypeptide, or the individual human calcium channel alpha1G-c domains. Specifically, it is readily apparent to those skilled in the art that monospecific antibodies may be generated which are specific for human calcium channel alpha1G-c by immunizing an animal with an antigenic peptide derived from the 23 amino acid insert, or fragments thereof.

Human calcium channel alpha1G-c antibody affinity columns are made by adding the antibodies to Affigel-10 (Bio-Rad), a gel support which is activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1M ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) and the cell culture supernatants or cell extracts containing human calcium channel alpha1G-c or human calcium channel alpha1G-c subunits are slowly passed through the column. The column is then washed with phosphate buffered saline until the optical density (A_{280}) falls to background, then the protein is eluted with 0.23 M glycine-HCl (pH 2.6). The purified human calcium channel alpha1G-c protein is then dialyzed against phosphate buffered saline.

DNA clones, termed human calcium channel alpha1G-c, are identified which encode proteins that, when expressed in a recombinant host cell, form channels that regulate calcium influx and are sensitive to NPPB (5-Nitro-2-(3-phenylpropylamino)benzoic acid). The expression of human calcium channel alpha1G-c DNA results in the reconstitution of the properties observed in oocytes injected with human calcium channel alpha1G-c-encoding poly $(A)^+$ RNA, including direct activation with the appropriate stimuli.

The present invention is also directed to methods for screening for compounds that modulate the expression of DNA or RNA encoding human calcium channel alpha1G-c as well as the quantity of expressed human calcium channel alpha1G-c protein. The term "compound" refers to small organic or inorganic molecules (including divalent ions), synthetic or natural amino acid polypeptides, proteins, or synthetic or natural nucleic acid sequences. Compounds may modulate by increasing or attenuating the expression of DNA or RNA encoding human calcium channel alpha1G-c, or the quantity of cell surface human calcium channel alpha1G-c protein. Compounds that modulate the expression of DNA or RNA encoding human calcium channel alpha1G-c or the quantity of human calcium channel alpha1G-c protein may be detected by a variety of assays. Assays to measure changes in the level of expression of alpha1G-c can be accomplished by various means, well known in the art, for example changes in the quantity of mRNA, intracellular protein (newly synthesize protein being processed within the endoplasmic reticulum or Golgi apparatus), or cell surface protein. Levels of mRNA are detected by reverse transcription polymerase chain reaction (RT-PCR) or by differential gene expression (quantitative gene chips). Immunoaffinity quantitates levels of protein both within and on the surface of host cells. Protein-specific affinity beads or specific antibodies are used to isolate for example ^{35}S-methionine labelled or unlabelled protein. Labelled protein is analyzed by SDS-PAGE. Unlabelled protein is detected by Western blotting, cell surface detection by fluorescent cell sorting, ELISA or RIA employing specific antibodies.

Assays that use eukaryotic cells for identifying compounds that modulate human alpha1G-c calcium channel activity are also provided. In practicing these assays the eukaryotic cell that expresses the heterologous human alpha1G-c calcium channel encoded by a DNA sequence described herein, is in a solution containing a test compound and a calcium channel selective ion, the cell membrane is depolarized, and current flowing into the cell is detected. If the test compound is one that modulates calcium channel activity, the current that is detected is different from that produced by depolarizing the same or a substantially identical cell in the presence of the same calcium channel-selective ion but in the absence of the compound. In preferred embodiments, the cells are mammalian cells, most preferably HEK293 cells, or amphibian oocytes. The assay method comprises the steps of: (a) measuring the activity of the human alpha1G-c in a cell that expresses the human alpha1G-c calcium channel; (b) contacting a compound with the cell; and (c) monitoring changes in the cell. In these assays, an agonist would increase Ca influx with no elevated K depolarizing stimulus, in the presence of concentrations of K that normally are not enough to activate the channels or shift the voltage dependence of inactivation. In these assays, an antagonist would block Ca influx induced by elevated potassium. Assays that measure electrophysiological calcium channel function measure the amount or duration of Ca

influx, for example by using Ca sensitive dyes such as Fluo-3 or radioactive ions such as ^{45}Ca or voltage clamp techniques. Voltage sensitive dyes and current clamp electrophysiological techniques can be used to measure depolarizations resulting from Ca influx. Yet another embodiment of the test method measures "downstream" effects of Ca influx by using a transcription based assay under inducible control of a Ca sensitive promotor, as described in PCT International Patent Application No. PCT/US91/5625, filed Aug. 7, 1991.

These assays may be a simple "yes/no" assays to determine whether there is a change in expression or function or they may be made quantitative by comparing the expression or function of a test sample with the levels of expression or function in a standard sample. Modulators identified any of these processes are useful as therapeutic agents.

Modulators identified in the assays disclosed herein are useful candidates as therapeutic agents for the treatment disorders that are mediated by human alpha1G-c activity. Such activities that may be mediated by human alpha1G-c include, epilepsy, schizophrenia, depression, sleep disorders, stress, endocrine disorders, respiratory disorder, peripheral muscle disorders, muscle excitability, Cushing's disease, fertilization, contraception, disorders involving neuronal firing regulation, respiratory disorders, hypertension, cardiac rhythm, potentiation of synaptic signals, improving arterial compliance in systolic hypertension, vascular tone such as by decreasing vascular swelling, cardiac hypertrophy, cardiac fibrosis, atherosclerosis, cardiovascular disorders, including but not limited to: myocardial infarct, cardiac arrhythmia, heart failure and angina pectoris, and cellular growth (protein synthesis, cell differentiation, and proliferation). The compounds that modulate human alpha1G-c calcium channel activity may be useful in regulating vascular smooth muscle tone, either vasodilating or vasoconstricting in: (a) treatments for reestablishing blood pressure control, e.g., following traumatic injury, surgery or cardiopulmonary bypass, and in prophylactic treatments designed to minimize cardiovascular effects of anaesthetic drugs; (b) treatments for improving vascular reflexes and blood pressure control by the autonomic nervous system. The compounds that modulate human alpha1G-c calcium channel activity may also be useful in treatments of urological disorders and reproductive disorders: (a) treating and restoring renal function following surgery, traumatic. injury, uremia and adverse drug reactions; (b) treating bladder dysfunctions; and (c) uremic neuronal toxicity and hypotension in patients on hemodialysis; reproductive disorders; (d) disorders of sexual function including impotence; (e) alcoholic impotence (under autonomic control that may be subject to T-type calcium channel controls); and (f) fertility (via direct action upon Sertoli cells (in males) or the zona pecullicda (for mammalian eggs) or by modulation of hormonal feedback). The compounds that modulate human alpha1G-c calcium channel activity may be useful in treatments of hepatic disorders in treating and reducing neuronal toxicity and autonomic nervous system damage resulting from acute overconsumption of alcohol. The compounds that modulate human alpha1G-c calcium channel activity may be useful treatments for neurologic disorders; (a) epilepsy and diencephalic epilepsy; (b) Parkinson's disease; and (c) aberrant temperature control, such as, abnormalities of shivering and sweat gland secretion and peripheral vascular blood supply. The compounds that modulate human alpha1G-c calcium channel activity may be useful for treating abnormal respiration, e.g., post-surgical complications of anesthetics and endocrine disorders; (a) aberrant pituitary and hypothalamic functions including abnormal secretion of noradrenaline, dopamine and other hormones; and (b) treatments for overproduction of insulin, thyroxine adrenaline and other hormonal imbalances.

Kits containing human calcium channel alpha1G-c DNA or RNA, antibodies to human calcium channel alpha1G-c, or human calcium channel alpha1G-c protein may be prepared. Such kits are used to detect DNA that hybridizes to human calcium channel alpha1G-c DNA or to detect the presence of human calcium channel alpha1G-c protein or peptide fragments in a sample. Such characterization is useful for a variety of purposes including but not limited to forensic analyses, diagnostic applications, and epidemiological studies.

The DNA molecules, RNA molecules, recombinant protein and antibodies of the present invention may be used to screen and measure levels of human calcium channel alpha1G-c DNA, human calcium channel alpha1G-c RNA or human calcium channel alpha1G-c protein. The recombinant proteins, DNA molecules, RNA molecules and antibodies lend themselves to the formulation of kits suitable for the detection and typing of human calcium channel alpha1G-c. Such a kit would comprise a compartmentalized carrier suitable to hold in close confinement at least one container. The carrier would further comprise reagents such as recombinant human calcium channel alpha1G-c protein or anti-human calcium channel alpha1G-c antibodies suitable for detecting human calcium channel alpha1G-c. The carrier may also contain a means for detection such as labeled antigen or enzyme substrates or the like.

Nucleotide sequences that are complementary to the human calcium channel alpha1G-c encoding DNA sequence can be synthesized for antisense therapy. These antisense molecules may be DNA, stable derivatives of DNA such as phosphorothioates or methylphosphonates, RNA, stable derivatives of RNA such as 2'-O-alkylRNA, or other human calcium channel alpha1G-c antisense oligonucleotide mimetics. Human calcium channel alpha1G-c antisense molecules may be introduced into cells by microinjection, liposome encapsulation or by expression from vectors harboring the antisense sequence. Human calcium channel alpha1G-c antisense therapy may be particularly useful for the treatment of diseases where it is beneficial to reduce human calcium channel alpha1G-c activity.

Human calcium channel alpha1G-c gene therapy may be used to introduce human calcium channel alpha1G-c into the cells of target organisms. The human calcium channel alpha1G-c gene can be ligated into viral vectors that mediate transfer of the human calcium channel alpha1G-c DNA by infection of recipient host cells. Suitable viral vectors include retrovirus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, polio virus and the like. Alternatively, human calcium channel alpha1G-c DNA can be transferred into cells for gene therapy by non-viral techniques including receptor-mediated targeted DNA transfer using ligand-DNA conjugates or adenovirus-ligand-DNA conjugates, lipofection membrane fusion or direct microinjection. These procedures and variations thereof are suitable for ex vivo as well as in vivo human calcium channel alpha1G-c gene therapy. Human calcium channel alpha1G-c gene therapy may be particularly useful for the treatment of diseases where it is beneficial to elevate human calcium channel alpha1G-c activity.

Pharmaceutically useful compositions comprising human calcium channel alpha1G-c DNA, human calcium channel

alpha1G-c RNA, or human calcium channel alpha1G-c protein, or modulators of human calcium channel alpha1G-c activity, may be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation may be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the protein, DNA, RNA, or modulator.

Therapeutic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat or diagnose disorders in which modulation of human calcium channel alpha1G-c-related activity is indicated. The effective amount may vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration. The pharmaceutical compositions may be provided to the individual by a variety of routes such as subcutaneous, topical, oral and intramuscular.

The term "chemical derivative" describes a molecule that contains additional chemical moieties that are not normally a part of the base molecule. Such moieties may improve the solubility, half-life, absorption, etc. of the base molecule. Alternatively the moieties may attenuate undesirable side effects of the base molecule or decrease the toxicity of the base molecule. Examples of such moieties are described in a variety of texts, such as Remington's Pharmaceutical Sciences.

Compounds identified according to the methods disclosed herein may be used alone at appropriate dosages defined by routine testing in order to obtain optimal inhibition of the human calcium channel alpha1G-c or its activity while minimizing any potential toxicity. In addition, co-administration or sequential administration of other agents may be desirable.

The present invention also has the objective of providing suitable topical, oral, systemic and parenteral pharmaceutical formulations for use in the novel methods of treatment of the present invention. The compositions containing compounds or modulators identified according to this invention as the active ingredient for use in the modulation of human calcium channel alpha1G-c receptors can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds or modulators can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they may also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts. An effective but non-toxic amount of the compound desired can be employed as a human calcium channel alpha1G-c modulating agent.

The daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per patient, per day. For oral administration, the compositions are preferably provided in the form of scored or unscored tablets containing 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, and 50.0 milligrams of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. An effective amount of the drug is ordinarily supplied at a dosage level of from about 0.0001 mg/kg to about 100 mg/kg of body weight per day. The range is more particularly from about 0.001 mg/kg to 10 mg/kg of body weight per day. The dosages of the human calcium channel alpha1G-c modulators are adjusted when combined to achieve desired effects. On the other hand, dosages of these various agents may be independently optimized and combined to achieve a synergistic result wherein the pathology is reduced more than it would be if either agent were used alone.

Advantageously, compounds or modulators of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three or four times daily. Furthermore, compounds or modulators for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

For combination treatment with more than one active agent, where the active agents are in separate dosage formulations, the active agents can be administered concurrently, or they each can be administered at separately staggered times.

The dosage regimen utilizing the compounds or modulators of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound thereof employed. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of drug within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the drug's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a drug.

In the methods of the present invention, the compounds or modulators herein described in detail can form the active ingredient, and are typically administered in admixture with suitable pharmaceutical diluents, excipients or carriers (collectively referred to herein as "carrier" materials) suitably selected with respect to the intended form of administration, that is, oral tablets, capsules, elixirs, syrups and the like, and consistent with conventional pharmaceutical practices.

For instance, for oral administration in the form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic pharmaceutically acceptable inert carrier such as ethanol, glycerol, water and the like. Moreover, when desired or necessary, suitable binders, lubricants, disintegrating agents and coloring agents can also be incorporated into the mixture. Suitable binders include, without limitation, starch, gelatin, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth or sodium alginate, carboxymethylcellulose, polyethylene glycol, waxes and the like. Lubricants used in these dosage forms include, without limitation, sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like. Disintegrators include, without limitation, starch, methyl cellulose, agar, bentonite, xanthan gum and the like.

For liquid forms the active drug component can be combined in suitably flavored suspending or dispersing agents such as the synthetic and natural gums, for example, tragacanth, acacia, methyl-cellulose and the like. Other dispersing agents that may be employed include glycerin and the like. For parenteral administration, sterile suspensions and solutions are desired. Isotonic preparations, which generally contain suitable preservatives, are employed when intravenous administration is desired.

Topical preparations containing the active drug component can be admixed with a variety of carrier materials well known in the art, such as, eg., alcohols, aloe vera gel, allantoin, glycerine, vitamin A and E oils, mineral oil, PPG2 myristyl propionate, and the like, to form, eg., alcoholic solutions, topical cleansers, cleansing creams, skin gels, skin lotions, and shampoos in cream or gel formulations.

The compounds or modulators of the present invention can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine or phosphatidylcholines.

Compounds of the present invention may also be delivered by the use of monoclonal antibodies as individual carriers to which the compound molecules are coupled. The compounds or modulators of the present invention may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinyl-pyrrolidone, pyran copolymer, polyhydroxypropylmethacryl-amidephenol, polyhydroxy-ethylaspartamidephenol, or polyethyleneoxidepolylysine substituted with palmitoyl residues. Furthermore, the compounds or modulators of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydro-pyrans, polycyanoacrylates and cross-linked or amphipathic block copolymers of hydrogels.

For oral administration, the compounds or modulators may be administered in capsule, tablet, or bolus form or alternatively they can be mixed in the animals feed. The capsules, tablets, and boluses are comprised of the active ingredient in combination with an appropriate carrier vehicle such as starch, talc, magnesium stearate, or di-calcium phosphate. These unit dosage forms are prepared by intimately mixing the active ingredient with suitable finely-powdered inert ingredients including diluents, fillers, disintegrating agents, and/or binders such that a uniform mixture is obtained. An inert ingredient is one that will not react with the compounds or modulators and which is non-toxic to the animal being treated. Suitable inert ingredients include starch, lactose, talc, magnesium stearate, vegetable gums and oils, and the like. These formulations may contain a widely variable amount of the active and inactive ingredients depending on numerous factors such as the size and type of the animal species to be treated and the type and severity of the infection. The active ingredient may also be administered as an additive to the feed by simply mixing the compound with the feedstuff or by applying the compound to the surface of the feed. Alternatively the active ingredient may be mixed with an inert carrier and the resulting composition may then either be mixed with the feed or fed directly to the animal. Suitable inert carriers include corn meal, citrus meal, fermentation residues, soya grits, dried grains and the like. The active ingredients are intimately mixed with these inert carriers by grinding, stirring, milling, or tumbling such that the final composition contains from 0.001 to 5% by weight of the active ingredient.

The compounds or modulators may alternatively be administered parenterally via injection of a formulation consisting of the active ingredient dissolved in an inert liquid carrier. Injection may be either intramuscular, intraluminal, intratracheal, or subcutaneous. The injectable formulation consists of the active ingredient mixed with an appropriate inert liquid carrier. Acceptable liquid carriers include the vegetable oils such as peanut oil, cotton seed oil, sesame oil and the like as well as organic solvents such as solketal, glycerol formal and the like. As an alternative, aqueous parenteral formulations may also be used. The vegetable oils are the preferred liquid carriers. The formulations are prepared by dissolving or suspending the active ingredient in the liquid carrier such that the final formulation contains from 0.005 to 10% by weight of the active ingredient.

Topical application of the compounds or modulators is possible through the use of a liquid drench or a shampoo containing the instant compounds or modulators as an aqueous solution or suspension. These formulations generally contain a suspending agent such as bentonite and normally will also contain an antifoaming agent. Formulations containing from 0.005 to 10% by weight of the active ingredient are acceptable. Preferred formulations are those containing from 0.01 to 5% by weight of the instant compounds or modulators.

The following examples illustrate the present invention without, however, limiting the same thereto.

EXAMPLE 1

Generation of a Human Thalamus Library cDNA Synthesis

First Strand Synthesis

Approximately 5 μg of human thalamus mRNA (Clontech) was used to synthesize cDNA using the cDNA synthesis kit (Life Technologies). Two microliters of Not1 primer adapter was added to 5 μl of mRNA and the mixture was heated to 70° C. for 10 minutes and placed on ice. The following reagents were added on ice: 4 μl of 5× first strand buffer (250 mM TRIS-HCl (pH8.3), 375 mM KCl, 15 mM $MgCl_2$), 2 μl of 0.1M DTT, 10 mM dNTP (nucleotide triphosphates) mix and 1 μl of DEPC treated water. The reaction was incubated at 42° C. for 5 minutes. Finally, 5 μl of Superscript RT II was added and incubated at 42° C. for 2 more hours. The reaction was terminated on ice.

Second Strand Synthesis

The first strand product was adjusted to 93 μl with water and the following reagents were added on ice: 30 μl of 5× 2nd strand buffer (5×concentration (in mM): 100 mM TRIS-HCl (pH6.9), 450 mM KCl, 23 mM $MgCl_2$, 0.75 mM β-NAD+, 50 mM $(NH_4)_2SO_4$), 3 μl of 10 mM dNTP (nucleotide triphosphates), 1 μl *E. coli* DNA ligase (10 units)1 μl RNase H (2 units), 4 μl DNA pol I (10 units)). The reaction was incubated at 16° C. for 2 hours. The DNA from second strand synthesis was treated with T4 DNA polymerase and placed at 16° C. to blunt the DNA ends. The double stranded cDNA was extracted with 150 μl of a mixture of phenol and chloroform (1:1, v:v) and precipitated with 0.5 volumes of 7.5 M NH4OAc and 2 volumes of absolute ethanol. The pellet was washed with 70% ethanol and dried down at 37° C. to remove the residual ethanol. The double stranded DNA pellet was resuspended in 25 μl of water and the following reagents were added; 10 μl of 5×T4 DNA ligase buffer, 10 μl of Sal1 adapters and 5 μl of T4 DNA ligase. The ingredients were mixed gently and ligated overnight at 16° C. The ligation mix was extracted with phenol:chloroform:isoamyl alcohol, vortexed thoroughly and centrifuged at room temperature for 5 minutes at

14,000xg to separate the phases. The aqueous phase was transferred to a new tube and the volume adjusted to 100 ml with water. The purified DNA was size selected on a chromaspin 1000 column (Clontech) to eliminate the smaller cDNA molecules. The double stranded DNA was digested with NotI restriction enzyme for 3–4 hours at 37° C. The restriction digest was electrophoresed on a 0.8% low melt agarose gel. The cDNA in the range of 1–5 kb was cut out and purified using Gelzyme (Invitrogen). The product was extracted with phenol:chloroform and precipitated with NH_4OAc and absolute ethanol. The pellet was washed with 70% ethanol and resuspended in 10 ml of water.

Ligation of cDNA to the Vector

The cDNA was split up into 5 tubes (2 μl each) and the ligation reactions were set up by adding 4.5 μl of water, 2 μl of 5xligation buffer, 1 μl of p-Sport vector DNA (cut with Sal-1/Not1 and phosphatase treated) and 0.5 μl of T4 DNA ligase. The ligation was incubated at 40° C. overnight.

Introduction of Ligated cDNA into *E.coli* by Electroporation

The ligation reaction volume was adjusted to a total volume of 20 μl with water. Five milliliters of yeast tRNA, 12.5 ml of 7.5M NH_4OAc and 70 ml of absolute ethanol (–20° C.) was added. The mixture was vortexed thoroughly, and immediately centrifuged at room temperature for 20 minutes at 14000xg. The pellets were washed in 70% ethanol and each pellet was resuspended in 5 ml of water. All 5 ligations (25 ml) were pooled and 100 μl of DH10B electro-competent cells (Life Technologies) were electroporated with 1 ml of DNA (total of 20 electroporations), then plated out on ampicillin plates to determine the number of recombinants (cfu) per microliter. The entire library was seeded into 2 liters of Super Broth and maxipreps were made using Promega Maxi Prep kit and purified on cesium chloride gradients.

EXAMPLE 2

Library Screening/Human Calcium Channel Alpha1G-c Generation

Human Thalamus Library Screening

One microliter aliquots of the human thalamus library were electroporated into Electromax DH10B cells (Life Technologies). The volume was adjusted to 1 ml with SOC media and incubated for 60 minutes at 37° C. with shaking. The library was then plated out on 150 cm^2 plates containing LB to a density of 20000 colonies per plate. These cultures were grown overnight at 37° C.

A human calcium channel alpha1G-c probe was generated by polymerase chain reaction using the following primer pair:

SEQ.ID.NO.:1 5' oligo (18341F) 5' GCACTGCCAGTGGC-CGAGGG

SEQ.IN.NO.:2 3' oligo (18747R): 5' _CCATGGCGATGGTGATGCAG

The probe was generated by PCR using regular PCR conditions using 5' and 3' probe oligos (bOOng each) and 10 ng of diluted miniprep DNA. The resulting 274 bp fragment was run on 1% agarose gel and purified using GENECLEAN kit (Bio 101, Inc.). About 100 ng of the purified probe was labeled with alpha 32P using oligolabeling kit from Pharmacia and the labeled DNA was purified with S-200 columns (Pharmacia).

The library colonies were lifted on Protran nitrocellulose filters (Scheicher & Schuel) and the DNA was denatured in 1.5 M NaCl, 0.5 M NaOH. The filter disks were neutralized with 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5 and then UV crosslinked to the membrane using a UV-Stratalinker (Stratagene). The filters were washed several times in wash solution (50 mM Tris-HCl, pH 8.0; 1 M NaCl; 1 mM EDTA; 0.1% SDS) at room temperature. Then the disks were incubated in 1xsouthern pre-hybridization buffer (5'-3' Inc) containing 50% formamide and 100 ug/ml of sheared salmon sperm DNA (5'-3' Inc) for 6 hours at 42 C. Finally, hybridization was performed overnight at 42 C. in 1xhybridization buffer (5'-3') containing 50% formamide, 100 ng of sheared salmon sperm DNA in the presence of labeled probe (5×10^5 to 1×10^6 cpm/ml of hybridization buffer).

The disks were washed once in 2xSSC, 0.2% SDS at room temperature for 30 minutes, once in 0.2xSSC, 0.1%SDS at 50 C. for 30 minutes, once in 0.2xSSC, 0.1%SDS at 55 C. for 30 minutes and once in 0.2xSSC, 0.1% SDS at 60 C. for 15 minutes. The membranes were than placed on sheets of filter paper, wrapped in the Saran Wrap and exposed to the film at –20 C. overnight.

Positive clones were identified and collected by coring the colonies from the original plate. The colonies were incubated in 2 ml of LB for 2 hours at 37° C. Dilutions of the cultures were plated onto LB agar plates and the filter-lifting, hybridizing, washing, colony-picking procedure was repeated. Individual clones from the second screen were picked and digested with EcoRI/NotI to determine the size of the inserts, and the inserts were sequenced.

Three different clones between 3–5kb in length were identified with open reading frames. These were digested with $EcoR_1$/Xho1 and Xho1/Not1. These two pieces that were 4.2 kb and 3.2 kb were subjected to a 3 way ligation using an aliquot of pSport-1 vector that was cut with EcoR1 and Not1 and purified on a low melting point agarose gel. The ligated circular plasmid DNA was transformed into DH5 alpha bacterial cells from Gibco BRL. A few clones were picked and the entire 7.4 kb sequence was reconfirmed. A maxiprep of the plasmid DNA was obtained using the Promega kit. This DNA was further digested with EcoR1 and Not 1 and the 7.4 kb was inserted into the expression vector pGEM HE . Large-scale preparation of DNA was done using a MEGA prep kit (Promega.).

EXAMPLE 3

Cloning Human Calcium Channel Alpha1G-c cDNA into a Mammalian Expression Vector

The human calcium channel alpha1G-c cDNAs (collectively referred to as hCaChalpha1G-c) were cloned into the mammalian expression vector pcDNA3.1/Zeo(+). The plasmid DNA in p-Sport vector was digested with Not I and EcoR1 (NEB) to create cohesive ends. The product was purified by a low melting agarose gel electrophoresis. The pcDNA3.1/Zeo(+) vector was digested with EcoR1 and Not1 enzymes and subsequently purified on a low melt agarose gel. The linear vector was used to ligate to the human calcium channel alpha1G-c cDNA inserts.

EXAMPLE 4

Construction of a Stable Cell Line Expressing the Human Alpha1G-c

Recombinants were isolated, designated human calcium channel alpha1G-c, and are used to transfect mammalian cells (HEK293, COS-7 or CHO-K1 cells) using the Effectene non-liposomal lipid based transfection kit (Quiagen). Stable cell clones are selected by growth in the presence of zeocin. Single zeocin resistant clones are isolated and shown to contain the intact human calcium channel alpha1G-c gene. Clones containing the human calcium channel alpha1G-c cDNAs are analyzed for human calcium channel alpha1G-cprotein expression. Recombinant plasmids containing human calcium channel alpha1G-c encoding DNA are used to transform the mammalian COS or CHO cells or HEK293 cells.

Cells expressing human calcium channel alpha1G-c, stably or transiently, are used to test for expression of human calcium channel alpha1G-c activity. These cells are used to identify and examine other compounds for their ability to modulate, inhibit or activate the human calcium channel alpha1G-c.

Cassettes containing the human calcium channel alpha1G-c cDNA in the positive orientation with respect to the promoter are ligated into appropriate restriction sites 3' of the promoter and identified by restriction site mapping and/or sequencing. These cDNA expression vectors are introduced into fibroblastic host cells for example COS-7 (ATCC# CRL1651), and CV-1 tat [Sackevitz et al., Science 238: 1575 (1987)], 293, L (ATCC# CRL6362)] by standard methods including but not limited to electroporation, or chemical procedures (cationic liposomes, DEAE dextran, calcium phosphate). Transfected cells and cell culture supernatants are harvested and analyzed for human calcium channel alpha1G-c expression as described herein.

All of the vectors used for mammalian transient expression can be used to establish stable cell lines expressing human calcium channel alpha1G-c. Unaltered human calcium channel alpha1G-c receptor cDNA constructs cloned into expression vectors are expected to program host cells to make human calcium channel alpha1G-c protein. The transfection host cells include, but are not limited to, CV-1-P [Sackevitz et al., Science 238: 1575 (1987)], tk-L [Wigler, et al. Cell 11: 223 (1977)], NS/0, and dHFr- CHO [Kaufmnan and Sharp, J. Mol. Biol. 159: 601, (1982)].

Human calcium channel alpha1G-c cDNA constructs are also ligated into vectors containing amplifiable drug-resistance markers for the production of mammalian cell clones synthesizing the highest possible levels of human calcium channel alpha1G-c. Following introduction of these constructs into cells, clones containing the plasmid are selected with the appropriate agent, and isolation of an over-expressing clone with a high copy number of plasmids is accomplished by selection in increasing doses of the agent.

Co-transfection of any vector containing human calcium channel alpha1G-c cDNA with a drug selection plasmid including, but not limited to G418, aminoglycoside phosphotransferase; hygromycin, hygromycin-B phospholransferase; APRT, xanthine-guanine phosphoribosyl-transferase or zeocin, will allow for the selection of stably transfected clones. Levels of human calcium channel alpha1G-c are quantitated by the assays described herein (EXAMPLE 6).

The expression of recombinant human calcium channel alpha1G-c is achieved by transfection of full-length human calcium channel alpha1G-c cDNA into a mammalian host cell.

EXAMPLE 5

Characterization of Functional Protein Encoded by pCaChalpha1G-c in Xenopus Oocytes

Xenopus laevis oocytes were prepared and injected using standard methods previously described and known in the art (Fraser, S. P. et al. (1993)). Ovarian lobes from adult female *Xenopus laevis* (Nasco, Fort Atkinson, Wis.) were teased apart, rinsed several times in nominally Ca-free saline containing: 82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, adjusted to pH 7.0 with NaOH (OR-2), and gently shaken in OR-2 containing 0.2% collagenase Type 1 (ICN Biomedicals, Aurora, Ohio) for 2–5 hours. When approximately 50% of the follicular layers were removed, Stage V and VI oocytes were selected and rinsed in media consisting of 75% OR-2 and 25% ND-96. The ND-96 contained: 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, 2.5 mM Na pyruvate, gentamicin (50 ug/ml), adjusted to pH 7.0 with NaOH. The extracellular Ca^{+2} was gradually increased and the cells were maintained in ND-96 for 2–24 hours before injection. For in vitro transcription, pGEM HE which had been modified to contain the multiple cloning site from pSPORT (Liman, E. R. et al. (1992)) containing human calcium channel alpha1G-c was linearized with NotI and transcribed with T7 RNA polymerase (Promega) in the presence of the cap analog m7G(5')ppp(5')G. The human alpha1G-c contained its natural Kozak sequence. The synthesized cRNA was precipitated with ammonium acetate and isopropanol, and resuspended in 50 μl nuclease-free water. cRNA was quantified using formaldehyde gels (1% agarose, 1×MOPS , 3% formaldehyde) against RNA markers (Gibco BRL, 0.24–9.5 Kb).

Oocytes were injected with 50 nl of the human calcium channel alpha1G-c cRNA (about 600 ng). Control oocytes were injected with 50 nl of water. Oocytes were incubated in ND-96 before analysis for expression of the human calcium channel alpha1G-c. Incubations and collagenase digestion were carried out at room temperature. Injected oocytes were maintained in 48 well cell culture clusters (Costar; Cambridge, Mass.) at 18° C. Whole cell agonist-induced currents were measured 3–6 days after injection with a conventional two-electrode voltage clamp (GeneClamp500, Axon Instruments, Foster City, Calif.) using standard methods previously described and known in the art (Dascal, N. (1987)). The microelectrodes were filled with 3 M KCl, which had resistances of 1 and 2 MΩ. Cells were continuously perfused with ND96 at 2–5 ml/min at room temperature unless indicated. In some experiments, cells were bathed in a 40 mM Ba saline containing (in mM): 40 $BaCl_2$, 2 KCl, 36 TEA-Cl, 5 4-AP and 5 HEPES, pH 7.6. Membrane voltage was clamped at −100 mV unless indicated.

Depolarizing voltage steps elicited inward currents in oocytes that had been injected with RNA transcribed from the cloned human calcium channel alpha1G-c cDNA as shown in FIGS. 4*a,b*. In some experiments in which oocytes expressed large outward currents and slowly activating inward currents at negative potentials (activation of endogenous Ca-activated Cl currents), oocytes were bathed in 40 mM Ba saline. Due to effects of Ba2+ on surface charge screening (Wilson et al., 1983), we usually used more physiological conditions (2 mM extracellular Ca^{2+}; ND96).

FIG. 4*a* shows a representative family of current traces elicited by depolarizing pulses applied to the oocyte. Inward Ba^{2+} currents activated slowly near threshold potentials and with larger depolarizing voltage pulses, the currents activated more quickly and inactivated, producing a signature "criss-cross" pattern for classical T-type currents (Randall and Tsien, 1997). Water-injected oocytes had no detectable inward currents. Peak currents recorded in 2 mM extracellular Ca^{2+} were −380+/−170 nA (n=9), similar to that observed with Ba^{2+} as the charge carrier (−240+/−20 nA; n=8). The threshold voltage recorded in ND96 was about −59 mV (n=9). The voltage that elicited maximal currents was −29+/−5 mV (n=9). The voltage at which currents reversed sign was +29+/−5 mV (n=4). The time to peak from the onset of the voltage pulse was 5.2+/−2 msec (n=9). In 40 mM Ba^{2+} solution, the voltage dependence of activation was shifted slightly along the voltage axis (Huguenard, 1996; Perez-Reyes et al., 1998). The voltage eliciting peak currents was −33+/−2 mV (n=8). The time to peak response was similar to that recorded in 2 mM Ca^{2+} (4.8+/−0.3 msec).

Steady state inactivation (FIGS. 4*c,d*) was studied by applying 4 sec long prepulses followed by a test pulse to −30

mV to measure channel availability. In some experiments a 5 msec repolarization pulse to −100 mV was performed to close any channels still open at the end of the 4 sec pulse. Results were similar and combined. Similar $V_{0.5}$ for inactivation for the cloned mouse alpha1G (AJ012569 contains the insert observed in the present invention in intracellular loop II-III as well as an extra 18 amino acid insert in intracellular loop between domains III–IV) expressed in HEK293 cells were obtained in Ca^{2+} and Ba^{2+} salines (Klugbauer et al., 1999). FIG. 4c shows representative current traces recorded during the test pulse. The percent of maximum response was calculated, plotted as a function of the prepulse potential and fit with a Boltzmann equation (FIG. 4*d*). Inactivation of human alpha1G-c occurred at sub-threshold voltages and displayed a steep voltage dependence (slope −4.9 [−6.0 to −3.8], n=7). The voltage dependence of inactivation occurred at −67 mV with 95% confidence interval of −68.3 to −65.8 mV (n=7 experiments; CaSOS). The voltage dependence of inactivation was similar when recorded in 40 mM Ba^{2+} (−71+/−5 mV, n=5).

A defining feature of T-type calcium currents is that they deactivate relatively slowly compared to HVA calcium currents, producing slowly decaying tail currents after a depolarizing pulse. A 5 msec voltage step to −30 mV was followed by a step to −100 mV. The tau for current deactivation was 2.2+/−0.4 msec (n=3), similar to values reported for T-type currents.

The pharmacological characterization of human alpha1G-c expressed in Xenopus oocytes was determined for mibefradil, Ni^{2+}, Cd^{2+}, amiloride and ethosuximide. The effect of the indicated concentrations of mibefradil on peak T-currents was determined. Mibefradil was bath applied to oocytes expressing human calcium channel alpha1G-c cRNA (FIG. 5). Shown are 1–3 concentrations tested on 7 individual oocytes. The IC_{50} was 2.5 μM with a 95% confidence interval of 1.3 to 4.9 μM. Oocytes were bathed in ND96,

The present invention was relatively insensitive to Ni^{2+} blockade, similar to that observed for the rat alpha1G (AJ027984) (Perez-Reyes et al., 1998). 200 μM $NiCl_2$ blocked the peak current by 25+/−6% (n=3); in the same cell, 1 mM $NiCl_2$ blocked about twice the current blocked by 200 μM Ni^{2+}. Oocytes were voltage clamped at −100 mV between test pulses. Cd^{2+} (100 μM) blocked T-currents by 44+/−9% (n=3).

The present invention was sensitive to amiloride block. 500 μM amiloride blocked peak currents by only 23+/−4% (n=4), similar to the block observed at rat spinal motoneurons (Huguenard, 1996). This concentration would completely block some T-type calcium currents (e.g., human alpha1H; see Background). Oocytes were maintained at −100 mV between voltage pulses and similar results were obtained for oocytes bathed in ND96 and Ba^{2+} salines.

The present invention was sensitive to block by the antiepileptic ethosuximide. 600 μM ethosuximide (Sigma), within the range of therapeutically relevant concentrations for the treatment of absence epilepsy (see Background), reversibly blocked peak currents by 26+/−−3% (n=3). Oocytes were maintained at −100 mV between voltage pulses and similar results were obtained for oocytes bathed in ND96 and Ba^{2+} salines. Human alpha1H currents are blocked only ~7% by 300 μM ethosuximide (WO 99/28342).

Interestingly, the chloride channel blocker NPPB (5-Nitro-2-(3-phenylpropylamino)benzoic acid) blocked human alpha1G-c currents expressed in oocytes. 20, 100 and 200 μM NPPB blocked 22+/−6% (n=3), 55+/−7% (n=3), and 89+/−7% (n=3), respectively. Another chloride channel blocker 9-AC (anthracene-9-carboxylic acid, Sigma) was less effective in blocking T-currents; 100 uM 9-AC blocked peak currents by 30+/−3% (n=4). DIDS (4,4'-diisothiocyanatostilbene-2.2'-disulfonic acid (Sigma); 100 μM) and niflumic acid (100 μM) had no effect on peak human alpha1G-c currents. DIDS and niflumic acid blocked the current by 16+/−13% (n=3) and 0+/−2% (n=3), respectively.

EXAMPLE 6

Characterization of Human Calcium Channel Alpha1G-c in Human HEK 293 Cell Line

Human HEK293 cells are transfected with human calcium channel alpha1G-c pCaChalpha1Gc (EXAMPLE 4). Transient transfections 1 μg of pCaChalpha1G per 10^6 cells per 100 mm dish are performed using the Effectene tranfection kit (Quiagen; 301425). Three days after transfection, cells are plated onto 96-well plates (Biocoat, poly-D-lysine coated black/clear plate; Becton Dickinson part #354640). After one day, wells are rinsed with F12/DMEM, then incubated in Fluo-4 (2 μM) with Pluronic acid (20%, 40 μl used in 20 mls total volume) for 1 hour at room temperature. Plates are assayed using the FLIPR (Molecular Devices, FL-101). Cells are challenged with elevated K+ to achieve a final concentrations of 10, 25 and 43 mM K^+ (applied in 40 μL added to 80 μl at a velocity of 50 μl/sec). Transfections with vector alone are tested as controls. The basal buffer contains (in mM): 123 NaCl, 2 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 15 glucose and 20 HEPES, pH 7.4.

Cells stably expressing the human alpha1G-c are plated onto 96-well plates (Biocoat, poly-D-lysine coated black/clear plate; Becton Dickinson part #354640) and grown to confluence. Wells are rinsed with F12/DMEM, then incubated in Fluo-4 (2 μM) with Pluronic acid (20%, 40 μl used in 20 mls total volume) for 1 hour at room temperature. Plates are assayed using the FLIPR (Molecular Devices, FL-101). Cells are challenged with elevated K+ (in 40 μl added to 80 μl at a velocity of 50 μl/sec).

The whole cell patch clamp technique (Hamill, O. P. et al. (1981)) is used to record ligand-induced currents from HEK293 stably expressing human calcium channel alpha1G-c maintained for >1 day on 12 mm coverslips. Cells are visualized using a Nikon Diaphot 300 with DIC Nomarski optics. Cells are continuously perfused in a physiological saline (~0.5 ml/min) unless otherwise indicated. The standard physiological saline ("CaCh physiological saline (CaChPS") contains: 15 mM BaC12, 150 mM CholineCl, 1 mM MgCl2 and 10 mM HEPES (pH 7.3, 325 mOsm as measured using a Wescor 5500 vapor-pressure (Wescor, Inc., Logan, Utah). Recording electrodes are fabricated from borosilicate capillary tubing (R6; Garner Glass, Claremont, Calif.), the tips are coated with dental periphery wax (Miles Laboratories, South Bend, Ind.), and have resistances of 1–2 MΩ when containing intracellular saline: 135 mM CsCl, 10 mM EGTA, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4, with TEA-OH, 290 mOsm). Current and voltage signals are detected and filtered at 2 kHz with an Axopatch ID patch-clamp amplifier (Axon Instruments, Foster City, Calif.), digitally recorded with a DigiData 1200B laboratory interface (Axon Instruents), and PC compatible computer system and stored on magnetic disk for off-line analysis. Data acquisition and analysis are performed with PClamp software.

EXAMPLE 7

Pimgar Structure Of the Human Calcium Channel Alpha1G-c Protein

The nucleotide sequences of human calcium channel alpha1G-c revealed single large open reading frame of about 6819 base pairs encoding 2273 amino acids. The cDNAs have 5' and 3'-untranslated extensions of about 511 and about 397 nucleotides for human calcium channel alpha1G-c, respectively. The first in-frame methionine was designated as the initiation codon for an open reading frame that predicts a human calcium channel alpha1G-c protein with an estimated molecular mass (M_r) of about 251.8 kDa.

The predicted human calcium channel alpha1G-c protein was aligned with nucleotide and protein databases and found to be similar to the human alpha1G "a" isoform (accession #AF126966) with the exception that the sequence presented herein contains a 23 amino acid insert in the second intracellular loop between domains I and II. The insert contains a putative CKII phosphorylation site at S971. This 23 amino acid insert is 91 and 87% identical to the homologous sequence in rat (AF125161) and mouse (AJ012569), respectively. However, this insert is not present in another rat alpha1G isoform (AF027984) which is the ortholog to the present invention in regard to the remainder of the sequence. The putative casein kinase II phosphorylation site in this insert in the present invention is not conserved in rat or mouse.

There are 8, 23, 15 and 12 putative PKA (ie., R/K R/K x T/S), PKC (ie., S/T×K/R), casein kinase II (CKII; ie. S/T xx D/E) and MGCK (mammary gland casein kinase; ie., S×E) phosphorylation sites, respectively. There are 8 potential N-linked glycosylation sites. There are no putative tyrosine phosphorylation motifs (i.e., R/K x x x D x x Y) in predicted intracellular domains.

EXAMPLE 8

Cloning Human Calcium Channel Alpha1G-c cDNA into *E. coli* Expression Vectors

Recombinant human calcium channel alpha1G-c is produced in *E. coli* following the transfer of the human calcium channel alpha1G-c expression cassette into *E. coli* expression vectors, including but not limited to, the pET series (Novagen). The pET vectors place human calcium channel alpha1G-c expression under control of the tightly regulated bacteriophage T7 promoter. Following transfer of this construct into an *E. coli* host that contain a chromosomal copy of the T7 RNA polymerase gene driven by the inducible lac promoter, expression of human calcium channel alpha1G-c is induced when an appropriate lac substrate (IPTG) is added to the culture. The levels of expressed human calcium channel alpha1G-c are determined by the assays described herein.

The cDNA encoding the entire open reading frame for human calcium channel alpha1G-c is inserted into the NdeI site of pET [16]11a. Constructs in the positive orientation are identified by sequence analysis and used to transform the expression host strain BL21. Transformants are then used to inoculate cultures for the production of human calcium channel alpha1G-c protein. Cultures may be grown in M9 or ZB media, whose formulation is known to those skilled in the art. After growth to an OD_{600}=1.5, expression of human calcium channel alpha1G-c is induced with 1 mM IPTG for 3 hours at 37° C.

EXAMPLE 9

Cloning Human Calcium Channel Alpha1G-c cDNA into a Baculovirus Expression Vector for Expression in Insect Cells

Baculovirus vectors, which are derived from the genome of the AcNPV virus, are designed to provide high level expression of cDNA in the Sf9 line of insect cells (ATCC CRL #1711). Recombinant baculoviruses expressing human calcium channel alpha1G-c cDNA is produced by the following standard methods (InVitrogen Maxbac Manual): the human calcium channel alpha1G-c cDNA constructs are ligated into the polyhedrin gene in a variety of baculovirus transfer vectors, including the pAC360 and the BlueBac vector (InVitrogen). Recombinant baculoviruses are generated by homologous recombination following co-transfection of the baculovirus transfer vector and linearized AcNPV genomic DNA [Kitts, P. A., Nuc. Acid. Res. 18: 5667 (1990)] into Sf9 cells. Recombinant pAC360 viruses are identified by the absence of inclusion bodies in infected cells and recombinant pBlueBac viruses are identified on the basis of β-galactosidase expression (Summers, M. D. and Smith, G. E., Texas Agriculture Exp. Station Bulletin No. 1555). Following plaque purification, human calcium channel alpha1G-c expression is measured by the assays described herein.

The cDNA encoding the entire open reading frame for human calcium channel alpha1G-c is inserted into the BamHI site of pBlueBacII. Constructs in the positive orientation are identified by sequence analysis and used to transfect Sf9 cells in the presence of linear AcNPV mild type DNA.

Authentic, active human calcium channel alpha1G-c is found in the cytoplasm of infected cells. Active human calcium channel alpha1G-c is extracted from infected cells by hypotonic or detergent lysis.

EXAMPLE 10

Cloning Human Calcium Channel Alpha1G-c cDNA into a Yeast Expression Vector

Recombinant human calcium channel alpha1G-c is produced in the yeast *S. cerevisiae* following insertion of the optimal human calcium channel alpha1G-c cDNA cistron into expression vectors designed to direct the intracellular or extracellular expression of heterologous proteins. In the case of intracellular expression, vectors such as EmBLyex4 or the like are ligated to the human calcium channel alpha1G-c cistron [Rinas, U. et al., Biotechnology 8: 543–545 (1990); Horowitz B. et al., J. Biol. Chem. 265: 4189–4192 (1989)]. For extracellular expression, the human calcium channel alpha1G-c cistron is ligated into yeast expression vectors which fuise a secretion signal (a yeast or mammalian peptide) to the NH_2 terminus of the human calcium channel alpha1G-c protein [Jacobson, M. A., Gene 85: 511–516 (1989); Riett L. and Bellon N. Biochem. 28: 2941–2949 (1989)].

These vectors include, but are not limited to pAVE1>6, which fuses the human serum albumin signal to the expressed cDNA [Steep 0. Biotechnology 8: 42–46 (1990)], and the vector pL8PL which fuses the human lysozyme signal to the expressed cDNA [Yamamoto, Y., Biochem. 28: 2728–2732)]. In addition, human calcium channel alpha1G-c is expressed in yeast as a fusion protein conjugated to ubiquitin utilizing the vector pVEP [Ecker, D. J., J. Biol. Chem. 264: 7715–7719 (1989), Sabin, E. A., Biotechnology 7: 705–709 (1989), McDonnell D. P., Mol. Cell Biol. 9: 5517–5523 (1989)]. The levels of expressed human calcium channel alpha1G-c are determined by the assays described herein.

EXAMPLE 11

Purification of Recombinant Human Calcium Channel Alpha1G-c

Recombinantly produced human calcium channel alpha1G-c may be purified by antibody affinity chromatography.

Human calcium channel alpha1G-c antibody affinity columns are made by adding the anti-human calcium channel alpha1G-c antibodies to Affigel-10 (Bio-Rad), a gel support that is pre-activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1M ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) together with appropriate membrane solubilizing agents such as detergents and the cell culture supernatant or cell extract containing solubilized human calcium channel alpha1G-c is slowly passed through the column. The column is then washed with phosphate-buffered saline together with detergents until the optical density (A280) falls to background, then the protein is eluted with 0.23 M glycine-HCl (pH 2.6) together with detergents. The purified human calcium channel alpha1G-c protein is then dialyzed against phosphate buffered saline.

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MET ASP GLU GLU GLU ASP GLY ALA GLY ALA GLU GLU SER GLY GLN PRO
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ARG SER PHE MET ARG LEU ASN ASP LEU SER GLY ALA GLY GLY ARG PRO
20 25 30

GLY PRO GLY SER ALA GLU LYS ASP PRO GLY SER ALA ASP SER GLU ALA
35 40 45

GLU GLY LEU PRO TYR PRO ALA LEU ALA PRO VAL VAL PHE PHE TYR LEU
50 55 60

SER GLN ASP SER ARG PRO ARG SER TRP CYS LEU ARG THR VAL CYS ASN
65 70 75 80

PRO TRP PHE GLU ARG ILE SER MET LEU VAL ILE LEU LEU ASN CYS VAL
85 90 95

THR LEU GLY MET PHE ARG PRO CYS GLU ASP ILE ALA CYS ASP SER GLN
100 105 110

ARG CYS ARG ILE LEU GLN ALA PHE ASP ASP PHE ILE PHE ALA PHE PHE
115 120 125

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ALA VAL GLU MET VAL VAL LYS MET VAL ALA LEU GLY ILE PHE GLY LYS
    130                 135                 140

LYS CYS TYR LEU GLY ASP THR TRP ASN ARG LEU ASP PHE PHE ILE VAL
145                 150                 155                 160

ILE ALA GLY MET LEU GLU TYR SER LEU ASP LEU GLN ASN VAL SER PHE
                165                 170                 175

SER ALA VAL ARG THR VAL ARG VAL LEU ARG PRO LEU ARG ALA ILE ASN
            180                 185                 190

ARG VAL PRO SER MET ARG ILE LEU VAL THR LEU LEU LEU ASP THR LEU
        195                 200                 205

PRO MET LEU GLY ASN VAL LEU LEU LEU CYS PHE PHE VAL PHE PHE ILE
    210                 215                 220

PHE GLY ILE VAL GLY VAL GLN LEU TRP ALA GLY LEU LEU ARG ASN ARG
225                 230                 235                 240

CYS PHE LEU PRO GLU ASN PHE SER LEU PRO LEU SER VAL ASP LEU GLU
                245                 250                 255

ARG TYR TYR GLN THR GLU ASN GLU ASP GLU SER PRO PHE ILE CYS SER
            260                 265                 270

GLN PRO ARG GLU ASN GLY MET ARG SER CYS ARG SER VAL PRO THR LEU
        275                 280                 285

ARG GLY ASP GLY GLY GLY GLY PRO PRO CYS GLY LEU ASP TYR GLU ALA
    290                 295                 300

TYR ASN SER SER SER ASN THR THR CYS VAL ASN TRP ASN GLN TYR TYR
305                 310                 315                 320

THR ASN CYS SER ALA GLY GLU HIS ASN PRO PHE LYS GLY ALA ILE ASN
                325                 330                 335

PHE ASP ASN ILE GLY TYR ALA TRP ILE ALA ILE PHE GLN VAL ILE THR
            340                 345                 350

LEU GLU GLY TRP VAL ASP ILE MET TYR PHE VAL MET ASP ALA HIS SER
        355                 360                 365

PHE TYR ASN PHE ILE TYR PHE ILE LEU LEU ILE ILE VAL GLY SER PHE
    370                 375                 380

PHE MET ILE ASN LEU CYS LEU VAL VAL ILE ALA THR GLN PHE SER GLU
385                 390                 395                 400

THR LYS GLN ARG GLU SER GLN LEU MET ARG GLU GLN ARG VAL ARG PHE
                405                 410                 415

LEU SER ASN ALA SER THR LEU ALA SER PHE SER GLU PRO GLY SER CYS
            420                 425                 430

TYR GLU GLU LEU LEU LYS TYR LEU VAL TYR ILE LEU ARG LYS ALA ALA
        435                 440                 445

ARG ARG LEU ALA GLN VAL SER ARG ALA ALA GLY VAL ARG VAL GLY LEU
    450                 455                 460

LEU SER SER PRO ALA PRO LEU GLY GLY GLN GLU THR GLN PRO SER SER
465                 470                 475                 480

SER CYS SER ARG SER HIS ARG ARG LEU SER VAL HIS HIS LEU VAL HIS
                485                 490                 495

HIS HIS HIS HIS HIS HIS HIS HIS TYR HIS LEU GLY ASN GLY THR LEU
            500                 505                 510

ARG ALA PRO ARG ALA SER PRO GLU ILE GLN ASP ARG ASP ALA ASN GLY
        515                 520                 525

SER ARG ARG LEU MET LEU PRO PRO PRO SER THR PRO ALA LEU SER GLY
    530                 535                 540
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ALA PRO PRO GLY GLY ALA GLU SER VAL HIS SER PHE TYR HIS ALA ASP
545                 550                 555                 560

CYS HIS LEU GLU PRO VAL ARG CYS GLN ALA PRO PRO PRO ARG SER PRO
                565                 570                 575

SER GLU ALA SER GLY ARG THR VAL GLY SER GLY LYS VAL TYR PRO THR
            580                 585                 590

VAL HIS THR SER PRO PRO PRO GLU THR LEU LYS GLU LYS ALA LEU VAL
        595                 600                 605

GLU VAL ALA ALA SER SER GLY PRO PRO THR LEU THR SER LEU ASN ILE
    610                 615                 620

PRO PRO GLY PRO TYR SER SER MET HIS LYS LEU LEU GLU THR GLN SER
625                 630                 635                 640

THR GLY ALA CYS GLN SER SER CYS LYS ILE SER SER PRO CYS LEU LYS
                645                 650                 655

ALA ASP SER GLY ALA CYS GLY PRO ASP SER CYS PRO TYR CYS ALA ARG
            660                 665                 670

ALA GLY ALA GLY GLU VAL GLU LEU ALA ASP ARG GLU MET PRO ASP SER
        675                 680                 685

ASP SER GLU ALA VAL TYR GLU PHE THR GLN ASP ALA GLN HIS SER ASP
    690                 695                 700

LEU ARG ASP PRO HIS SER ARG ARG GLN ARG SER LEU GLY PRO ASP ALA
705                 710                 715                 720

GLU PRO SER SER VAL LEU ALA PHE TRP ARG LEU ILE CYS ASP THR PHE
                725                 730                 735

ARG LYS ILE VAL ASP SER LYS TYR PHE GLY ARG GLY ILE MET ILE ALA
            740                 745                 750

ILE LEU VAL ASN THR LEU SER MET GLY ILE GLU TYR HIS GLU GLN PRO
        755                 760                 765

GLU GLU LEU THR ASN ALA LEU GLU ILE SER ASN ILE VAL PHE THR SER
    770                 775                 780

LEU PHE ALA LEU GLU MET LEU LEU LYS LEU LEU VAL TYR GLY PRO PHE
785                 790                 795                 800

GLY TYR ILE LYS ASN PRO TYR ASN ILE PHE ASP GLY VAL ILE VAL VAL
                805                 810                 815

ILE SER VAL TRP GLU ILE VAL GLY GLN GLN GLY GLY GLY LEU SER VAL
            820                 825                 830

LEU ARG THR PHE ARG LEU MET ARG VAL LEU LYS LEU VAL ARG PHE LEU
        835                 840                 845

PRO ALA LEU GLN ARG GLN LEU VAL VAL LEU MET LYS THR MET ASP ASN
    850                 855                 860

VAL ALA THR PHE CYS MET LEU LEU MET LEU PHE ILE PHE ILE PHE SER
865                 870                 875                 880

ILE LEU GLY MET HIS LEU PHE GLY CYS LYS PHE ALA SER GLU ARG ASP
                885                 890                 895

GLY ASP THR LEU PRO ASP ARG LYS ASN PHE ASP SER LEU LEU TRP ALA
            900                 905                 910

ILE VAL THR VAL PHE GLN ILE LEU THR GLN GLU ASP TRP ASN LYS VAL
        915                 920                 925

LEU TYR ASN GLY MET ALA SER THR SER SER TRP ALA ALA LEU TYR PHE
    930                 935                 940

ILE ALA LEU MET THR PHE GLY ASN TYR VAL LEU PHE ASN LEU LEU VAL
945                 950                 955                 960

ALA ILE LEU VAL GLU GLY PHE GLN ALA GLU GLU ILE SER LYS ARG GLU
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965 970 975

ASP ALA SER GLY GLN LEU SER CYS ILE GLN LEU PRO VAL ASP SER GLN
980 985 990

GLY GLY ASP ALA ASN LYS SER GLU SER GLU PRO ASP PHE PHE SER PRO
995 1000 1005

SER LEU ASP GLY ASP GLY ASP ARG LYS LYS CYS LEU ALA LEU VAL SER
1010 1015 1020

LEU GLY GLU HIS PRO GLU LEU ARG LYS SER LEU LEU PRO PRO LEU ILE
1025 1030 1035 1040

ILE HIS THR ALA ALA THR PRO MET SER LEU PRO LYS SER THR SER THR
1045 1050 1055

GLY LEU GLY GLU ALA LEU GLY PRO ALA SER ARG ARG THR SER SER SER
1060 1065 1070

GLY SER ALA GLU PRO GLY ALA ALA HIS GLU MET LYS SER PRO PRO SER
1075 1080 1085

ALA ARG SER SER PRO HIS SER PRO TRP SER ALA ALA SER SER TRP THR
1090 1095 1100

SER ARG ARG SER SER ARG ASN SER LEU GLY ARG ALA PRO SER LEU LYS
1105 1110 1115 1120

ARG ARG SER PRO SER GLY GLU ARG ARG SER LEU LEU SER GLY GLU GLY
1125 1130 1135

GLN GLN SER GLN ASP GLN GLU GLU SER SER GLU GLU GLU ARG ALA SER
1140 1145 1150

PRO ALA GLY SER ASP HIS ARG HIS ARG GLY SER LEU GLU ARG GLU ALA
1155 1160 1165

LYS SER SER PHE ASP LEU PRO ASP THR LEU GLN VAL PRO GLY LEU HIS
1170 1175 1180

ARG THR ALA SER GLY ARG GLY SER ALA SER GLU HIS GLN ASP CYS ASN
1185 1190 1195 1200

GLY LYS SER ALA SER GLY ARG LEU ALA ARG ALA LEU ARG PRO ASP ASP
1205 1210 1215

PRO PRO LEU ASP GLY ASP ASP ALA ASP ASP GLU GLY ASN LEU SER LYS
1220 1225 1230

GLY GLU ARG VAL ARG ALA TRP ILE ARG ALA ARG LEU PRO ALA CYS CYS
1235 1240 1245

LEU GLU ARG ASP SER TRP SER ALA TYR ILE PHE PRO PRO GLN SER ARG
1250 1255 1260

PHE ARG LEU LEU CYS HIS ARG ILE ILE THR HIS LYS MET PHE ASP HIS
1265 1270 1275 1280

VAL VAL LEU VAL ILE ILE PHE LEU ASN CYS ILE THR ILE ALA MET GLU
1285 1290 1295

ARG PRO LYS ILE ASP PRO HIS SER ALA GLU ARG ILE PHE LEU THR LEU
1300 1305 1310

SER ASN TYR ILE PHE THR ALA VAL PHE LEU ALA GLU MET THR VAL LYS
1315 1320 1325

VAL VAL ALA LEU GLY TRP CYS PHE GLY GLU GLN ALA TYR LEU ARG SER
1330 1335 1340

SER TRP ASN VAL LEU ASP GLY LEU LEU VAL LEU ILE SER VAL ILE ASP
1345 1350 1355 1360

ILE LEU VAL SER MET VAL SER ASP SER GLY THR LYS ILE LEU GLY MET
1365 1370 1375

LEU ARG VAL LEU ARG LEU LEU ARG THR LEU ARG PRO LEU ARG VAL ILE
1380 1385 1390

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SER ARG ALA GLN GLY LEU LYS LEU VAL VAL GLU THR LEU MET SER SER
        1395                1400                1405

LEU LYS PRO ILE GLY ASN ILE VAL VAL ILE CYS CYS ALA PHE PHE ILE
    1410                1415                1420

ILE PHE GLY ILE LEU GLY VAL GLN LEU PHE LYS GLY LYS PHE PHE VAL
1425                1430                1435                1440

CYS GLN GLY GLU ASP THR ARG ASN ILE THR ASN LYS SER ASP CYS ALA
                1445                1450                1455

GLU ALA SER TYR ARG TRP VAL ARG HIS LYS TYR ASN PHE ASP ASN LEU
            1460                1465                1470

GLY GLN ALA LEU MET SER LEU PHE VAL LEU ALA SER LYS ASP GLY TRP
        1475                1480                1485

VAL ASP ILE MET TYR ASP GLY LEU ASP ALA VAL GLY VAL ASP GLN GLN
    1490                1495                1500

PRO ILE MET ASN HIS ASN PRO TRP MET LEU LEU TYR PHE ILE SER PHE
1505                1510                1515                1520

LEU LEU ILE VAL ALA PHE PHE VAL LEU ASN MET PHE VAL GLY VAL VAL
                1525                1530                1535

VAL GLU ASN PHE HIS LYS CYS ARG GLN HIS GLN GLU GLU GLU GLU ALA
            1540                1545                1550

ARG ARG ARG GLU GLU LYS ARG LEU ARG ARG LEU GLU LYS LYS ARG ARG
        1555                1560                1565

SER LYS GLU LYS GLN MET ALA GLU ALA GLN CYS LYS PRO TYR TYR SER
    1570                1575                1580

ASP TYR SER ARG PHE ARG LEU LEU VAL HIS HIS LEU CYS THR SER HIS
1585                1590                1595                1600

TYR LEU ASP LEU PHE ILE THR GLY VAL ILE GLY LEU ASN VAL VAL THR
                1605                1610                1615

MET ALA MET GLU HIS TYR GLN GLN PRO GLN ILE LEU ASP GLU ALA LEU
            1620                1625                1630

LYS ILE CYS ASN TYR ILE PHE THR VAL ILE PHE VAL LEU GLU SER VAL
        1635                1640                1645

PHE LYS LEU VAL ALA PHE GLY PHE ARG ARG PHE PHE GLN ASP ARG TRP
    1650                1655                1660

ASN GLN LEU ASP LEU ALA ILE VAL LEU LEU SER ILE MET GLY ILE PRO
1665                1670                1675                1680

LEU GLU GLN ILE GLU VAL ASN ALA SER LEU PRO ILE ASN PRO THR ILE
                1685                1690                1695

ILE ARG ILE MET ARG VAL LEU ARG ILE ALA ARG VAL LEU LYS LEU LEU
            1700                1705                1710

LYS MET ALA VAL GLY MET ARG ALA LEU LEU ASP THR VAL MET GLN ALA
        1715                1720                1725

LEU PRO GLN VAL GLY ASN LEU GLY LEU LEU PHE MET LEU LEU PHE PHE
    1730                1735                1740

ILE PHE ALA ALA LEU GLY VAL GLU LEU PHE GLY ASP LEU GLU CYS ASP
1745                1750                1755                1760

GLU THR HIS PRO CYS GLU GLY LEU GLY ARG HIS ALA THR PHE ARG ASN
                1765                1770                1775

PHE GLY MET ALA PHE LEU THR LEU PHE ARG VAL SER THR GLY ASP ASN
            1780                1785                1790

TRP ASN GLY ILE MET LYS ASP THR LEU ARG ASP CYS ASP GLN GLU SER
        1795                1800                1805
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THR CYS TYR ASN THR VAL ILE SER PRO ILE TYR PHE VAL SER PHE VAL
   1810                1815                1820

LEU THR ALA GLN PHE VAL LEU VAL ASN VAL VAL ILE ALA VAL LEU MET
1825                1830                1835                1840

LYS HIS LEU GLU GLU SER ASN LYS GLU ALA LYS GLU GLU ALA GLU LEU
               1845                1850                1855

GLU ALA GLU LEU GLU LEU GLU MET LYS THR LEU SER PRO GLN PRO HIS
           1860                1865                1870

SER PRO LEU GLY SER PRO PHE LEU TRP PRO GLY VAL GLU GLY PRO ASP
       1875                1880                1885

SER PRO ASP SER PRO LYS PRO GLY ALA LEU HIS PRO ALA ALA HIS ALA
   1890                1895                1900

ARG SER ALA SER HIS PHE SER LEU GLU HIS PRO THR MET GLN PRO HIS
1905                1910                1915                1920

PRO THR GLU LEU PRO GLY PRO ASP LEU LEU THR VAL ARG LYS SER GLY
               1925                1930                1935

VAL SER ARG THR HIS SER LEU PRO ASN ASP SER TYR MET CYS ARG HIS
           1940                1945                1950

GLY SER THR ALA GLU GLY PRO LEU GLY HIS ARG GLY TRP GLY LEU PRO
       1955                1960                1965

LYS ALA GLN SER GLY SER VAL LEU SER VAL HIS SER GLN PRO ALA ASP
   1970                1975                1980

THR SER TYR ILE LEU GLN LEU PRO LYS ASP ALA PRO HIS LEU LEU GLN
1985                1990                1995                2000

PRO HIS SER ALA PRO THR TRP GLY THR ILE PRO LYS LEU PRO PRO PRO
               2005                2010                2015

GLY ARG SER PRO LEU ALA GLN ARG PRO LEU ARG ARG GLN ALA ALA ILE
           2020                2025                2030

ARG THR ASP SER LEU ASP VAL GLN GLY LEU GLY SER ARG GLU ASP LEU
       2035                2040                2045

LEU ALA GLU VAL SER GLY PRO SER PRO PRO LEU ALA ARG ALA TYR SER
   2050                2055                2060

PHE TRP GLY GLN SER SER THR GLN ALA GLN GLN HIS SER ARG SER HIS
2065                2070                2075                2080

SER LYS ILE SER LYS HIS MET THR PRO PRO ALA PRO CYS PRO GLY PRO
               2085                2090                2095

GLU PRO ASN TRP GLY LYS GLY PRO PRO GLU THR ARG SER SER LEU GLU
           2100                2105                2110

LEU ASP THR GLU LEU SER TRP ILE SER GLY ASP LEU LEU PRO PRO GLY
       2115                2120                2125

GLY GLN GLU GLU PRO PRO SER PRO ARG ASP LEU LYS LYS CYS TYR SER
   2130                2135                2140

VAL GLU ALA GLN SER CYS GLN ARG ARG PRO THR SER TRP LEU ASP GLU
2145                2150                2155                2160

GLN ARG ARG HIS SER ILE ALA VAL SER CYS LEU ASP SER GLY SER GLN
               2165                2170                2175

PRO HIS LEU GLY THR ASP PRO SER ASN LEU GLY GLY GLN PRO LEU GLY
           2180                2185                2190

GLY PRO GLY SER ARG PRO LYS LYS LYS LEU SER PRO PRO SER ILE THR
       2195                2200                2205

ILE ASP PRO PRO GLU SER GLN GLY PRO ARG THR PRO PRO SER PRO GLY
   2210                2215                2220

ILE CYS LEU ARG ARG ARG ALA PRO SER SER ASP SER LYS ASP PRO LEU
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2225                2230                2235                2240

ALA SER GLY PRO PRO ASP SER MET ALA ALA SER PRO SER PRO LYS LYS
                2245                2250                2255

ASP VAL LEU SER LEU SER GLY LEU SER SER ASP PRO ALA ASP LEU ASP
            2260                2265                2270

PRO
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What is claimed is:

1. An isolated and purified DNA molecule that encodes human calcium channel alpha1G-c channel protein, comprising an amino acid sequence set forth in SEQ ID NO: 5.

2. The isolated and purified DNA molecule of claim 1, having a nucleotide sequence selected from a group consisting of SEQ ID NO: 3 and SEQ ID NO: 4.

3. An expression vector for expression of a human calcium channel alpha1G-c channel protein in a recombinant host, wherein said vector contains a recombinant nucleic acid molecule encoding human calcium channel alpha1G-c protein comprising an amino acid sequence set forth in SEQ ID NO: 5.

4. The expression vector of claim 3, wherein the expression vector contains a cloned nucleic acid molecule encoding human calcium channel alpha1G-c channel protein having a nucleotide sequence selected from a group consisting of SEQ ID NO: 3 and SEQ ID NO: 4.

5. A process for expression of human calcium channel alpha1G-c channel protein in a recombinant host cell, comprising:

(a) transferring the expression vector of claim 3 into suitable host cells; and

(b) culturing the host cells of step (a) under conditions which allow expression of the human calcium channel alpha1G-c channel protein from the expression vector.

6. A recombinant host cell containing a recombinantly cloned nucleic acid molecule encoding human calcium channel alpha1G-c channel protein comprising an amino acid sequence set forth in SEQ ID NO: 5.

7. The recombinant host cell of claim 6, wherein said nucleic acid molecule has a nucleotide sequence selected from a group consisting of SEQ ID NO: 3 and SEQ ID NO: 4.

* * * * *

US006309858B1

(12) **United States Patent**
Dietrich et al.

(10) **Patent No.: US 6,309,858 B1**
(45) **Date of Patent: Oct. 30, 2001**

(54) **T-TYPE CALCIUM CHANNEL VARIANTS; COMPOSITIONS THEREOF; AND USES**

(75) Inventors: **Paul Shartzer Dietrich**, Palo Alto; **Joseph Gerard McGivern**, Mountain View, both of CA (US)

(73) Assignee: **Syntex (U.S.A.) LLC**, Palo Alto, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/404,650**

(22) Filed: **Sep. 23, 1999**

Related U.S. Application Data

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(51) **Int. Cl.**[7] **C12P 21/02**; C12N 5/00; C12N 15/00; C07H 21/02; C07H 21/04

(52) **U.S. Cl.** **435/69.1**; 435/320.1; 435/325; 435/335; 435/455; 536/23.1; 536/23.5

(58) **Field of Search** 530/23.1, 23.5; 435/320.1, 325, 69.1, 335

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(57) **ABSTRACT**

The invention provides TCCV-1 or TCCV-2 from human, reagents related thereto including polynucleotides encoding TCCV-1 or TCCV-2, purified polypeptides, and specific antibodies. Methods of making and using these reagents, in particular, methods for screening compounds which modulate TCCV-1 or TCCV-2 activity are provided. Also provided are methods of diagnosis and kits.

12 Claims, 10 Drawing Sheets

Figure 1A

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SEQ ID NO:2    MAESASPPSSSAAAPAAEPGVTTEQPGPRSPPSSPPGLEEPLDGADPHVPHPDLAPIAFF   60
SEQ ID NO:4    MAESASPPSSSAAAPAAEPGVTTEQPGPRSPPSSPPGLEEPLDGADPHVPHPDLAPIAFF   60
SEQ ID NO:5    MADSNLPPSS.AAAPAPEPGI.TEQPGPRSPPPSPPGLEEPLEGTNPDVPHPDLAPVAFF   58

SEQ ID NO:2    CLRQTTSPRNWCIKMVCNPWFECVSMLVILLNCVTLGMYQPCDDMDCLSDRCKILQVFDD   120
SEQ ID NO:4    CLRQTTSPRNWCIKMVCNPWFECVSMLVILLNCVTLGMYQPCDDMDCLSDRCKILQVFDD   120
SEQ ID NO:5    CLRQTTSPRNWCIKMVCNPWFECVSMLVILLNCVTLGMYQPCDDMECLSDRCKILQVFDD   118

SEQ ID NO:2    FIFIFFAMEMVLKMVALGIFGKKCYLGDTWNRLDFFIVMAGMVEYSLDLQNINLSAIRTV   180
SEQ ID NO:4    FIFIFFAMEMVLKMVALGIFGKKCYLGDTWNRLDFFIVMAGMVEYSLDLQNINLSAIRTV   180
SEQ ID NO:5    FIFIFFAMEMVLKMVALGIFGKKCYLGDTWNRLDFFIVMAGMVEYSLDLQNINLSAIRTV   178

SEQ ID NO:2    RVLRPLKAINRVPSMRILVNLLLDTLPMLGNVLLLCFFVFFIFGIIGVQLWAGLLRNRCF   240
SEQ ID NO:4    RVLRPLKAINRVPSMRILVNLLLDTLPMLGNVLLLCFFVFFIFGIIGVQLWAGLLRNRCF   240
SEQ ID NO:5    RVLRPLKAINRVPSMRILVNLLLDTLPMLGNVLLLCFFVFFIFGIIGVQLWAGLLRNRCF   238

SEQ ID NO:2    LEENFTIQGDVALPPYYQPEEDDEMPFICSLSGDNGIMGCHEIPPLKEQGRECCLSKDDV   300
SEQ ID NO:4    LEENFTIQGDVALPPYYQPEEDDEMPFICSLSGDNGIMGCHEIPPLKEQGRECCLSKDDV   300
SEQ ID NO:5    LEENFTIQGDVALPPYYQPEEDDEMPFICSLTGDNGIMGCHEIPPLKEQGRECCLSKDDV   298

SEQ ID NO:2    YDFGAGRQDLNASGLCVNWNRYYNVCRTGSANPHKGAINFDNIGYAWIVIFQVITLEGWV   360
SEQ ID NO:4    YDFGAGRQDLNASGLCVNWNRYYNVCRTGSANPHKGAINFDNIGYAWIVIFQVITLEGWV   360
SEQ ID NO:5    YDFGAGRQDLNASGLCVNWNRYYNVCRTGNANPHKGAINFDNIGYAGIVIFQVITLEGWV   358
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Figure 1B

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SEQ ID NO:2    EIMYYVMDAHSFYNFIYFILLIIVGSFFMINLCLVVIATQFSETKQREHRLMLEQRQRYL  420
SEQ ID NO:4    EIMYYVMDAHSFYNFIYFILLIIVGSFFMINLCLVVIATQFSETKQREHRLMLEQRQRYL  420
SEQ ID NO:5    EIMYYVMDAHSFYNFIYFILLIIVGSFFMINLCLVVIATQFSETKQREHRLMLEQRQRYL  418

SEQ ID NO:2    SSSTVASYAEPGDCYEEIFQYVCHILRKAKRRALGLYQALQSRRQALGPEAPAPAKPGPH  480
SEQ ID NO:4    SSSTVASYAEPGDCYEEIFQYVCHILRKAKRRALGLYQALQSRRQALGPEAPAPAKPGPH  480
SEQ ID NO:5    SSSTVASYAEPGDCYEEIFQYVCHILRKAKRRALGLYQALQNRRQAMGPGTPAPAKPGPH  478

SEQ ID NO:2    AKEPRHYQLCPQHSPLDATPHTLVQPIPATLASDPASCPCCQHEDGRRPSGLGSTDSGQE  540
SEQ ID NO:4    AKEPRHYQLCPQHSPLDATPHTLVQPIPATLASDPASCPCCQHEDGRRPSGLGSTDSGQE  540
SEQ ID NO:5    AKEPSHCKLCPRHSPLDPTPHTLVQPISAILASDPSSCPHCQHEAGRRPSGLGSTDSGQE  538

SEQ ID NO:2    GSGSGSSAGGEDEADGDGARSSEDGASSELGKEEEEEEQADGAVWLCGDVWRETRAKLRG  600
SEQ ID NO:4    GSGSGSSAGGEDEADGDGARSSEDGASSELGKEEEEEEQADGAVWLCGDVWRETRAKLRG  600
SEQ ID NO:5    GSGSGGSA..EAEANGDGLQSSEDGVSSDLGKEE...EQEDGAARLCGDVWRETRKKLRG  593

SEQ ID NO:2    IVDSKYFNRGIMMAILVNTVSMGIEHHEQPEELTNILEICNVVFTSMFALEMILKLAAFG  660
SEQ ID NO:4    IVDSKYFNRGIMMAILVNTVSMGIEHHEQPEELTNILEICNVVFTSMFALEMILKLAAFG  660
SEQ ID NO:5    IVDSKYFNRGIMMAILVNTVSMGIEHHEQPEELTNILEICNVVFTSMFALEMILKLAAFG  653

SEQ ID NO:2    LFDYLRNPYNIFDSIIVIISIWEIVGQADGGLSVLRTFRLLRVLKLVRFMPALRRQLVVL  720
SEQ ID NO:4    LFDYLRNPYNIFDSIIVIISIWEIVGQADGGLSVLRTFRLLRVLKLVRFMPALRRQLVVL  720
SEQ ID NO:5    LFDYLRNPYNIFDSIIVIISIWEIVGQADGGLSVLRTFRLLRVLKLVRFMPALRRQLVVL  713
```

Figure 1C

SEQ ID NO:2	MKTMDNVATFCMLLMLFIFIFSILGMHIFGCKFSLRTDTGDTVPDRKNFDSLLWAIVTVF	780
SEQ ID NO:4	MKTMDNVATFCMLLMLFIFIFSILGMHIFGCKFSLRTDTGDTVPDRKNFDSLLWAIVTVF	780
SEQ ID NO:5	MKTMDNVATFCMLLMLFIFIFSILGMHIFGCKFSLRTDTGDTVPDRKNFDSLLWAIVTVF	773
SEQ ID NO:2	QILTQEDWNVVLYNGMASTSPWASLYFVALMTFGNYVLFNLLVAILVEGFQAEGDANRSY	840
SEQ ID NO:4	QILTQEDWNVVLYNGMASTSPWASLYFVALMTFGNYVLFNLLVAILVEGFQAEGDANRSY	840
SEQ ID NO:5	QILTQEDWNVVLYNGMASTTPWASLYFVALMTFGNYVLFNLLVAILVEGFQAEGDANRSC	833
SEQ ID NO:2	SDEDQSSSNIEEFDKLQEGLDSSGDPKLCPIPMTPNGHLDPSLPLGGHLGPAGAAGPAPR	900
SEQ ID NO:4	SDEDQSSSNIEEFDKLQEGLDSSGDPKLCPIPMTPNGHLDPSLPLGGHLGPAGAAGPAPR	900
SEQ ID NO:5	SDEDQSSSNLEEFDKLPEGLDNSRDLKLCPIPMTPNGHLDPSLPLGAHLGPAGTMGTAPR	893
SEQ ID NO:2	LSLQPDPMLVALGSRKSSVMSLGRMSYDQRSLSSSRSSYYGPWGRSAAWASRRSSWNSLK	960
SEQ ID NO:4	LSLQPDPMLVALGSRKSSVMSLGRMSYDQRSLSSSRSSYYGPWGRSAAWASRRSSWNSLK	960
SEQ ID NO:5	LSLQPDPVLVALDSRKSSVMSLGRMSYDQRSLSSSRSSYYGPWGRSGTWASRRSSWNSLK	953
SEQ ID NO:2	HKPPSAEHESLLSAERGGG.ARVCEVAADEGPPRAAPLHTPHAHHIHHGPHLAHRHRHHR	1019
SEQ ID NO:4	HKPPSAEHESLLSAERGGG.ARVCEVAADEGPPRAAPLHTPHAHHIHHGPHLAHRHRHHR	1019
SEQ ID NO:5	HKPPSAEHESLLSGEGGGSCVRACEGAREEAPTRTAPLHAPHAHHAHHGPHLAHRHRHHR	1013
SEQ ID NO:2	RTLSLDNRDSVDLAELVPAVGAHPRAAWRAAGPAPGHEDCNGRMPSIAKDVFTKMGDRGD	1079
SEQ ID NO:4	RTLSLDNRDSVDLAELVPAVGAHPRAAWRAAGPAPGHEDCNGRMPSIAKDVFTKMGDRGD	1079
SEQ ID NO:5	RTLSLDTRDSVDLGELVPVVGAHSRAAWRGAGQAPGHEDCNGRMPNIAKDVFTKMDDRRD	1073

Figure 1D

SEQ ID NO:2	RGEDEEEIDYTLCFRVRKMIDVYKPDWCEVREDWSVYLFSPENRFRVLCQTIIAHKLFDY	1139
SEQ ID NO:4	RGEDEEEIDYTLCFRVRKMIDVYKPDWCEVREDWSVYLFSPENRFRVLCQTIIAHKLFDY	1139
SEQ ID NO:5	RGEDEEEIDYTLCFRVRKMIDVYKPDWCEVREDWSVYLFSPEN**K**FR**I**LCQTIIAHKLFDY	1133
SEQ ID NO:2	VVLAFIFLNCITIALERPQIEAGSTERIFLTVSNYIFTAIFVGEMTLKVVSLGLYFGEQA	1199
SEQ ID NO:4	VVLAFIFLNCITIALERPQIEAGSTERIFLTVSNYIFTAIFVGEMTLKVVSLGLYFGEQA	1199
SEQ ID NO:5	VVLAFIFLNCITIALERPQIEAGSTERIFLTVSNYIFTAIFVGEMTLKVVSLGLYFGEQA	1199
SEQ ID NO:2	YLRSSWNVLDGFLVFVSIIDIVVSLASAGGAKILGVLRVLRLLRTLRPLRVISRAPGLKL	1259
SEQ ID NO:4	YLRSSWNVLDGFLVFVSIIDIVVSLASAGGAKILGVLRVLRLLRTLRPLRVISRAPGLKL	1259
SEQ ID NO:5	YLRSSWNVLDGFLVFVSIIDIVVS**V**ASAGGAKILGVLRVLRLLRTLRPLRVISRAPGLKL	1253
SEQ ID NO:2	VVETLISSLKPIGNIVLICCAFFIIFGILGVQLFKGKFYHCLGVDTRNITNRSDCMAANY	1319
SEQ ID NO:4	VVETLISSLKPIGNIVLICCAFFIIFGILGVQLFKGKFYHCLGVDTRNITNRSDCMAANY	1319
SEQ ID NO:5	VVETLISSLKPIGNIVLICCAFFIIFGILGVQLFKGKFYHCLGVDTRNITNRSDC**V**AANY	1313
SEQ ID NO:2	RWVHHKYNFDNLGQALMSLFVLASKDGWVNIMYNGLDAVAVDQQPVTNHNPWMLLYFISF	1379
SEQ ID NO:4	RWVHHKYNFDNLGQALMSLFVLASKDGWVNIMYNGLDAVAVDQQPVTNHNPWMLLYFISF	1379
SEQ ID NO:5	RWVHHKYNFDNLGQALMSLFVLASKDGWVNIMYNGLDAVAVDQQPVTNHNPWMLLYFISF	1373
SEQ ID NO:2	LLIVSFFVLNMFVGVVVENFHKCRQHQEAEEARRREEKRLRRLEKKRRKAQRLPYYATYC	1439
SEQ ID NO:4	LLIVSFFVLNMFVGVVVENFHKCRQHQEAEEARRREEKRLRRLEKKRRKAQRLPYYATYC	1439
SEQ ID NO:5	LLIVSFFVLNMFVGVVVENFHKCRQHQEAEEARRREEKRLRRLEKKRRKAQRLPYYATYC	1433

Figure 1E

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SEQ ID NO:2    HTRLLIHSMCTSHYLDIFITFIICLNVVTMSLEHYNQPTSLETALKYCNYMFTTVFVLEA 1499
SEQ ID NO:4    HTRLLIHSMCTSHYLDIFITFIICLNVVTMSLEHYNQPTSLETALKYCNYMFTTVFVLEA 1499
SEQ ID NO:5    PTRLLIHSMCTSHYLDIFITFIICLNVVTMSLEHYNQPTSLETALKYCNYMFTTVFVLEA 1493

SEQ ID NO:2    VLKLVAFGLRRFFKDRWNQLDLAIVLLSVMGITLEEIEINAALPINPTIIRIMRVLRIAR 1559
SEQ ID NO:4    VLKLVAFGLRRFFKDRWNQLDLAIVLLSVMGITLEEIEINAALPINPTIIRIMRVLRIAR 1559
SEQ ID NO:5    VLKLVAFGLRRFFKDRWNQLDLAIVLLSVMGITLEEIEINAALPINPTIIRIMRVLRIAR 1553

SEQ ID NO:2    VLKLLKMATGMRALLDTVVQALPQVGNLGLLFMLLFFIYAALGVELFGKLVCNDENPCEG 1619
SEQ ID NO:4    VLKLLKMATGMRALLDTVVQALPQVGNLGLLFMLLFFIYAALGVELFGKLVCNDENPCEG 1619
SEQ ID NO:5    VLKLLKMATGMRALLDTVVQALPQVGNLGLLFMLLFFIYAALGVELFGKLVCNDENPCEG 1613

SEQ ID NO:2    MSRHATFENFGMAFLTLFQVSTGDNWNGIMKDTLRDCTHDERSCLSSLQFVSPLYFVSFV 1679
SEQ ID NO:4    MSRHATFENFGMAFLTLFQVSTGDNWNGIMKDTLRDCTHDERSCLSSLQFVSPLYFVSFV 1679
SEQ ID NO:5    MSRHATFENFGMAFLTLFQVSTGDNWNGIMKDTLRDCTHDERTCLSSLQFVSPLYFVSFV 1673

SEQ ID NO:2    LTAQFVLINVVVAVLMKHLDDSNKEAQEDAEMDAELELEMAHGLGP..............  1725
SEQ ID NO:4    LTAQFVLINVVVAVLMKHLDDSNKEAQEDAEMDAELELEMAHGLGP..............  1725
SEQ ID NO:5    LTAQFVLINVVVAVLMKHLDDSNKEAQEDAEMDAEIELEMAHGLGPCPGPCPGPCPCPCP 1733

SEQ ID NO:2    ....GPRLPTGSPGAPGRGPGGAGGGGDTEGGLCRRCYSPAQ.............DSLEG 1768
SEQ ID NO:4    ....GPRLPTGSPGAPGRGPGGAGGGGDTEGGLCRRCYSPAQENLWLDSVSLIIKDSLEG 1781
SEQ ID NO:5    CPCAGPRLPTSSPGAPGRGSGGAGAGGDTESHLCRHCYSPAQETLWLDSVSLIIKDSLEG 1793
```

Figure 1F

SEQ ID NO:2	ELTIIDNLSGSIFHHYSSPAGCKKCHHDKQEVQLAETEAFSLNSDRSSSILLGDDLSLED	1828
SEQ ID NO:4	ELTIIDNLSGSIFHHYSSPAGCKKCHHDKQEVQLAETEAFSLNSDRSSSILLGDDLSLED	1841
SEQ ID NO:5	ELTIIDNLSGS**V**FHHY**A**SP**D**GC**G**KCHHDKQE**TGLHPSCWGMT**	1835
SEQ ID NO:2	PTACPPGRKDSKGELDPPEPMRVGDLGECFFPLSSTAVSPDPENFLCEMEEIPFNPVRSW	1888
SEQ ID NO:4	PTACPPGRKDSKGELDPPEPMRVGDLGECFFPLSSTAVSPDPENFLCEMEEIPFNPVRSW	1901
SEQ ID NO:2	LKHDSSQAPPSPFSPDASSPLLPMPAEFFHPAVSASQKGPEKGTGTGTLPKIALQGSWAS	1948
SEQ ID NO:4	LKHDSSQAPPSPFSPDASSPLLPMPAEFFHPAVSASQKGPEKGTGTGTLPKIALQGSWAS	1961
SEQ ID NO:2	LRSPRVNCTLLRQATGSDTSLDASPSSSAGSLQTTLEDSLTLSDSPRRALGPPAPAPGPR	2008
SEQ ID NO:4	LRSPRVNCTLLRQATGSDTSLDASPSSSAGSLQTTLEDSLTLSDSPRRALGPPAPAPGPR	2021
SEQ ID NO:2	AGLSPAARRRLSLRGRGLFSLRGLRAHQRSHSSGGSTSPGCTHHDSMDPSDEEGRGGAGG	2068
SEQ ID NO:4	AGLSPAARRRLSLRGRGLFSLRGLRAHQRSHSSGGSTSPGCTHHDSMDPSDEEGRGGAGG	2081
SEQ ID NO:2	GGAGSEHSETLSSLSLTSLFCPPPPPPAPGLTPARKFSSTSSLAAPGRPHAAALAHGLAR	2128
SEQ ID NO:4	GGAGSEHSETLSSLSLTSLFCPPPPPPAPGLTPARKFSSTSSLAAPGRPHAAALAHGLAR	2141
SEQ ID NO:2	SPSWAADRSKDPPGRAPLPMGLGPLAPPPQPLPGELEPGDAASKRKR	2175
SEQ ID NO:4	SPSWAADRSKDPPGRAPLPMGLGPLAPPPQPLPGELEPGDAASKRKR	2188

Figure 2A

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                                  30 ↓ 31
SEQ ID NO:1     5148   ATGAAGGACACGCTGCGGGACTGCACCCACGACGAGCGCAGCTGCCTGAGCAGCCTGCAG
SEQ ID NO:3     5148   ATGAAGGACACGCTGCGGGACTGCACCCACGACGAGCGCAGCTGCCTGAGCAGCCTGCAG
SEQ ID NO:12    4927   ATGAAGGACACCCTGCGAGACTGTACCCATGATGAGCGCACGTGCCTAAGCAGCCTGCAG
                                                          CCTGAGCAGCCTGCAG

SEQ ID NO:1     5208   TTTGTGTCGCCGCTGTACTTCGTGAGCTTCGTGCTCACCGCGCAGTTCGTGCTCATCAAC
SEQ ID NO:3     5208   TTTGTGTCGCCGCTGTACTTCGTGAGCTTCGTGCTCACCGCGCAGTTCGTGCTCATCAAC
SEQ ID NO:12    4987   TTTGTGTCACCGCTCTACTTTGTGAGCTTCGTGCTCACAGCTCAGTTCGTGCTCATCAAC
                       TTTGTGT→6066

SEQ ID NO:1     5268   GTGGTGGTGGCTGTGCTCATGAAGCACCTGGACGACAGCAACAAGGAGGCGCAGGAGGAC
SEQ ID NO:3     5268   GTGGTGGTGGCTGTGCTCATGAAGCACCTGGACGACAGCAACAAGGAGGCGCAGGAGGAC
SEQ ID NO:12    5047   GTGGTGGTGGCCGTGCTGATGAAACATCTGGATGACAGCAACAAGGAGGCCCAGGAGGAT

SEQ ID NO:1     5328   GCCGAGATGGATGCCGAGCTCGAGCTGGAGATGGCCCATGGCCTGGGCCC----------
SEQ ID NO:3     5328   GCCGAGATGGATGCCGAGCTCGAGCTGGAGATGGCCCATGGCCTGGGCCC----------
SEQ ID NO:12    5107   GCAGAGATGGATGCTGAGATCGAGCTGGAGATGGCCCATGGCCTCGGCCCCTGCCCTGGC

SEQ ID NO:1     5378   ------------------------------------------TGGCCCGAGGCTGCCT
SEQ ID NO:3     5378   ------------------------------------------TGGCCCGAGGCTGCCT
SEQ ID NO:12    5167   CCCTGCCCTGGTCCCTGCCCCTGCCCCTGCCCCTGCCCCTGTGCTGGCCCGAGGCTGCCC

SEQ ID NO:1     5394   ACCGGCTCCCCGGGCGCCCCTGGCCGAGGGCCGGGAGGGGCGGGCGGCGGGGGCGACACC
SEQ ID NO:3     5394   ACCGGCTCCCCGGGCGCCCCTGGCCGAGGGCCGGGAGGGGCGGGCGGCGGGGGCGACACC
SEQ ID NO:12    5227   ACTAGTTCACCTGGGGCTCCGGGGCGAGGATCGGGAGGGGCAGGTGCTGGAGGCGACACC
```

Figure 2B

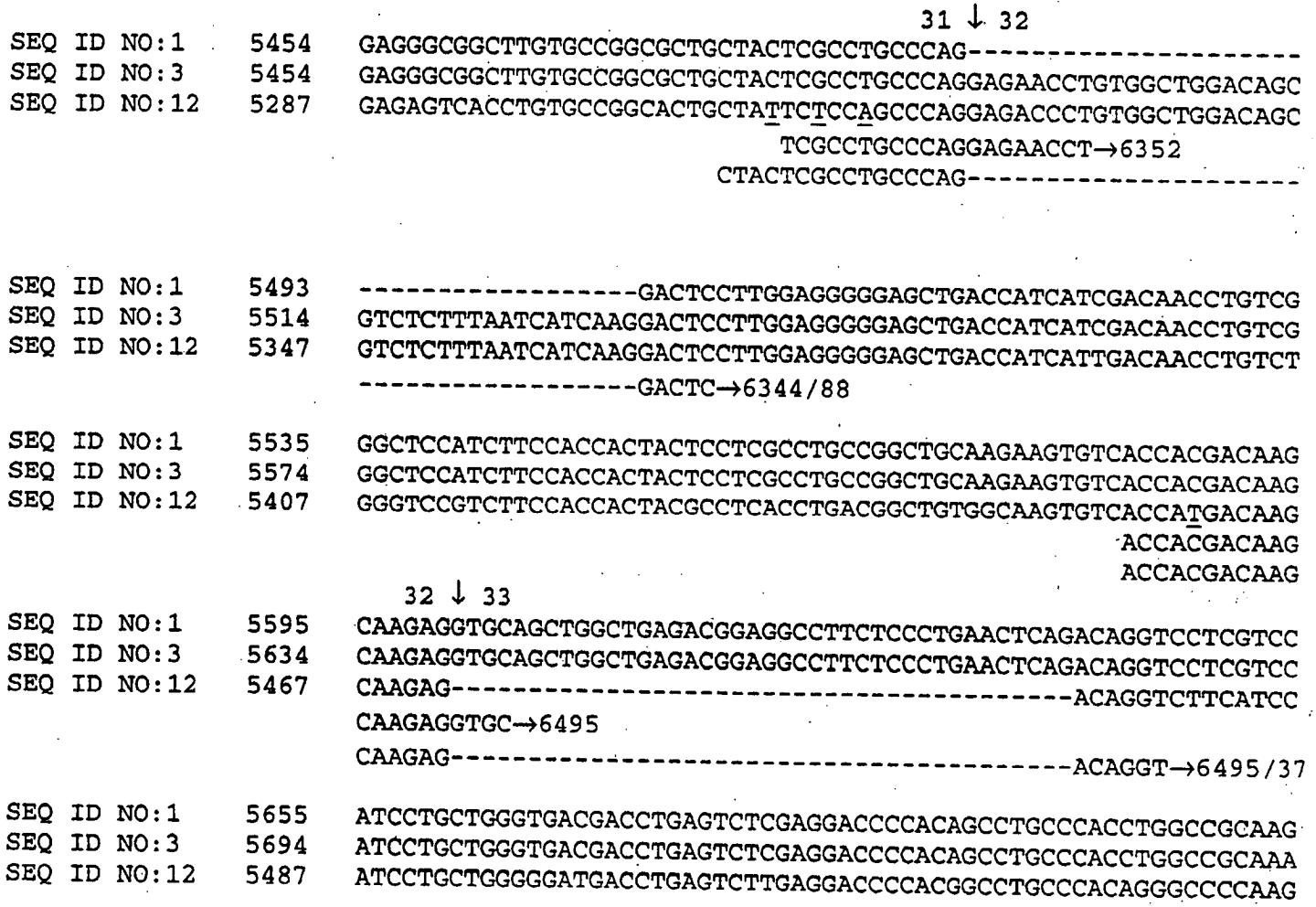
31 ↓ 32
SEQ ID NO:1 5454 GAGGGCGGCTTGTGCCGGCGCTGCTACTCGCCTGCCCAG----------
SEQ ID NO:3 5454 GAGGGCGGCTTGTGCCGGCGCTGCTACTCGCCTGCCCAGGAGAACCTGTGGCTGGACAGC
SEQ ID NO:12 5287 GAGAGTCACCTGTGCCGGCACTGCTATTCTCCAGCCCAGGAGACCCTGTGGCTGGACAGC
TCGCCTGCCCAGGAGAACCT→6352
CTACTCGCCTGCCCAG----------
SEQ ID NO:1 5493 ----------GACTCCTTGGAGGGGGAGCTGACCATCATCGACAACCTGTCG
SEQ ID NO:3 5514 GTCTCTTTAATCATCAAGGACTCCTTGGAGGGGGAGCTGACCATCATCGACAACCTGTCG
SEQ ID NO:12 5347 GTCTCTTTAATCATCAAGGACTCCTTGGAGGGGGAGCTGACCATCATTGACAACCTGTCT
----------GACTC→6344/88
SEQ ID NO:1 5535 GGCTCCATCTTCCACCACTACTCCTCGCCTGCCGGCTGCAAGAAGTGTCACCACGACAAG
SEQ ID NO:3 5574 GGCTCCATCTTCCACCACTACTCCTCGCCTGCCGGCTGCAAGAAGTGTCACCACGACAAG
SEQ ID NO:12 5407 GGGTCCGTCTTCCACCACTACGCCTCACCTGACGGCTGTGGCAAGTGTCACCATGACAAG
ACCACGACAAG
ACCACGACAAG
32 ↓ 33
SEQ ID NO:1 5595 CAAGAGGTGCAGCTGGCTGAGACGGAGGCCTTCTCCCTGAACTCAGACAGGTCCTCGTCC
SEQ ID NO:3 5634 CAAGAGGTGCAGCTGGCTGAGACGGAGGCCTTCTCCCTGAACTCAGACAGGTCCTCGTCC
SEQ ID NO:12 5467 CAAGAG----------ACAGGTCTTCATCC
CAAGAGGTGC→6495
CAAGAG----------ACAGGT→6495/37
SEQ ID NO:1 5655 ATCCTGCTGGGTGACGACCTGAGTCTCGAGGACCCCACAGCCTGCCCACCTGGCCGCAAG
SEQ ID NO:3 5694 ATCCTGCTGGGTGACGACCTGAGTCTCGAGGACCCCACAGCCTGCCCACCTGGCCGCAAA
SEQ ID NO:12 5487 ATCCTGCTGGGGGATGACCTGAGTCTTGAGGACCCCACGGCCTGCCCACAGGGCCCCAAG

Figure 2C

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                                  33 ↓ 34
SEQ ID NO:1     5715   GACAGCAAGGGTGAGCTGGACCCACCTGAGCCCATGCGTGTGGGAGACCTGGGCGAATGC
SEQ ID NO:3     5754   GACAGCAAGGGTGAGCTGGACCCACCTGAGCCCATGCGTGTGGGAGACCTGGGCGAATGC
SEQ ID NO:12    5547   GAGAGCAAGGGTGAACTAGAGCCTCCGGAGCCCATGCAGGCTGGAGACCTGGATGAATGC

SEQ ID NO:1     5775   TTCTTCCCCTTGTCC-TCTACGGCCGTCTCGCCGGATCCAGAGAACTTCCTGTGTGAGATG
SEQ ID NO:3     5814   TTCTTCCCCTTGTCC-TCTACGGCCGTCTCGCCGGATCCAGAGAACTTCCTGTGTGAGATG
SEQ ID NO:12    5607   TTTTGGCCCTTTGCCAAGCGAGCCAGTGTCCACAGGCCCAGAGAGCCTGCTGTGCGAGATG

                                                              34 ↓ 35
SEQ ID NO:1     5835   GAGGAGATCCCATTCAACCCTGTCCGGTCCTGGCTGAAACATGACAGCAGTCAAGCACCC
SEQ ID NO:3     5874   GAGGAGATCCCATTCAACCCTGTCCGGTCCTGGCTGAAACATGACAGCAGTCAAGCACCC
SEQ ID NO:12    5667   GGGGCCATTCCATTCAACCCTGTCCAGTCCTGGCTCAAACACGAGAGCAGCCAAGCACCC

SEQ ID NO:1     5895   CCAAGTCCCTTCTCCCCGGATGCCTCCAGCCCTCTCCTGCCCATGCCAGCCGAGTTCTTC
SEQ ID NO:3     5934   CCAAGTCCCTTCTCCCCGGATGCCTCCAGCCCTCTCCTGCCCATGCCAGCCGAGTTCTTC
SEQ ID NO:12    5727   CAGAGCCCTTTCTCCCCGGATGGCTCCAGCCCTCTCCTGTAGATGCCTGCTGAGTTCTTC
                                                    6831 ←CTCAAGAAG

SEQ ID NO:1     5955   CACCCTGCAGTGTCTGCCAGCCAGAAAGGCCCAGAAAAGGGCACTGGCACTGGAACCCTC
SEQ ID NO:3     5994   CACCCTGCAGTGTCTGCCAGCCAGAAAGGCCCAGAAAAGGGCACTGGCACTGGAACCCTC
SEQ ID NO:12    5787   CACCCTGCTGTGTCTGCCAGCCAGAAGGGGCAGGAACCGGGCATGAGTGCAGGAACCCTG
                       GTGGGACGTCACAGAC
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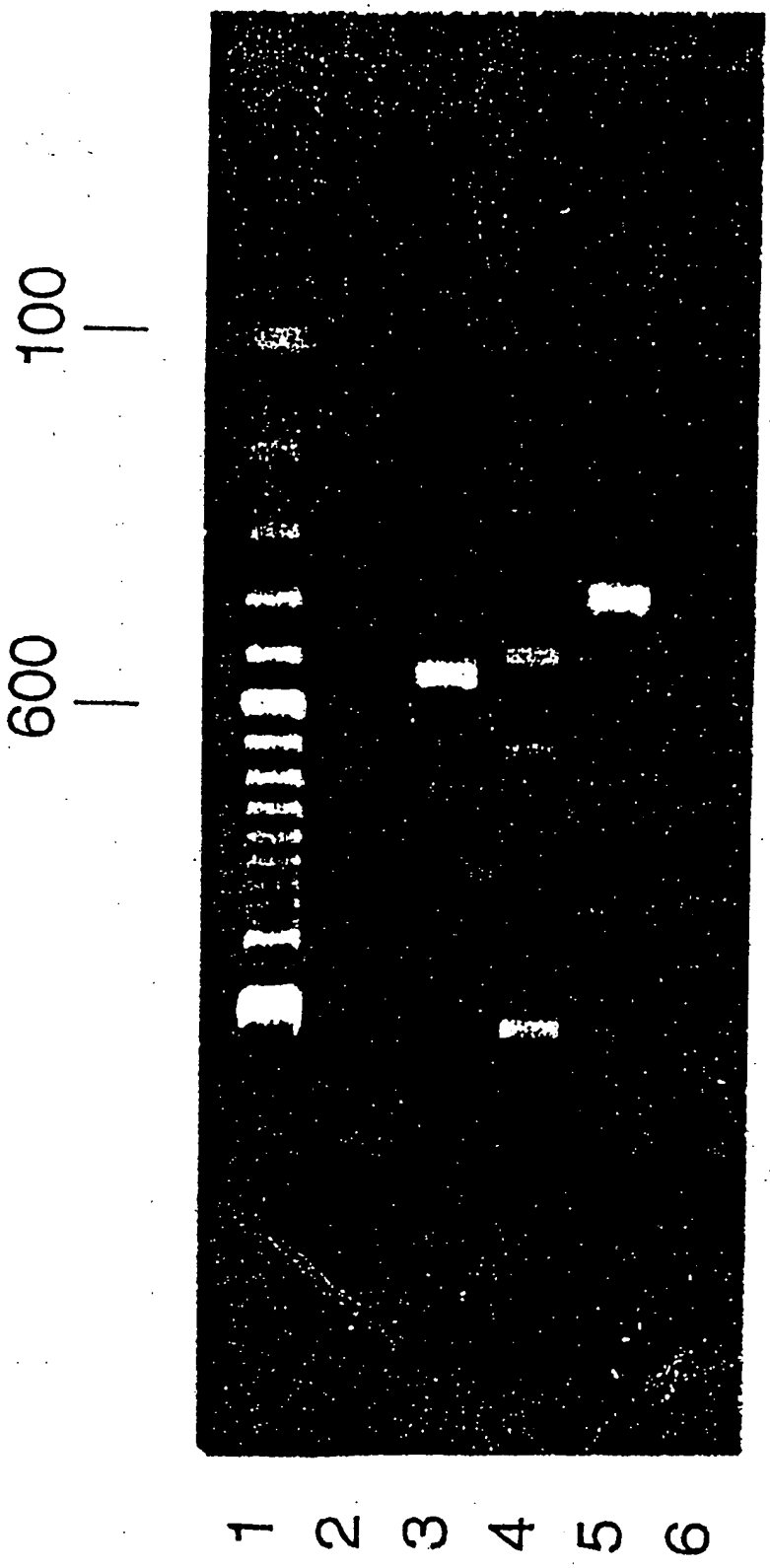

FIGURE 3

T-TYPE CALCIUM CHANNEL VARIANTS; COMPOSITIONS THEREOF; AND USES

RELATED APPLICATIONS

This U.S. patent application claims priority under 35 U.S.C. 119(e) to U.S. Provisional Application Serial No. 60/102,222, filed Sep. 29, 1998, incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to nucleic acid and amino acid sequences of human T-type calcium channel variants and the use of these sequences in diagnosis of disease states associated with pain and for use as targets for screening therapeutic compounds useful in the treatment of disease states associated with pain.

BACKGROUND OF THE INVENTION

Voltage-gated calcium channels can be divided into high- and low-threshold types. The high-threshold channels include the dihydropyridine-sensitive L-type, the ω-conotoxin GVIA-sensitive N-type and ω-agatoxin IVA-sensitive P-type. Depending on the tissue, these channel subtypes consist of α_1, $\alpha_2\delta$, β and γ subunits. (Perez-Reyes and Schneider (1995) Kid. Int. 48:1111–1124.) To date, only one type of low-threshold calcium channel is known, the T-type calcium channel.

T-type calcium channels have hyperpolarized steady-state inactivation characteristics, a low threshold for inactivation, small single channel conductance and display rapid inactivation kinetics. (Ertel and Ertel (1997) Trends Pharmacol. Sci. 18:37–42.) The functional roles for T-type calcium channels in neurons include membrane depolarization, calcium entry and burst firing. (White et al. (1989) Proc. Natl. Acad. Sci. USA 86:6802–6806.) T-type calcium channels are found in many neurons of the central and peripheral nervous systems, including small and medium diameter neurons of the dorsal root ganglia (Scroggs and Fox (1992) J. Physiol. 445:639–658) and neurons in the thalamus. (Suzuki and Rogawski (1989) Proc. Natl. Acad. Sci. USA 86:7228–7232.)

Calcium currents have been found to be important in several neurological and muscular functions, e.g., pain transmission, cardiac pacemaker activity, etc. Improper functioning of these channels has been implicated in arrythmias, chronic peripheral pain, improper pain transmission in the central nervous system, and epilepsy.

Anti-epileptic drugs are known to cause a reduction of the low-threshold calcium current (LTCC or T-type Ca^{2+} current) in thalamic neurons. (Coulter et al.(1989) Ann. Neurol. 25:582–593.) One such anti-epileptic compound, ethosuximide, has been shown to fully block T-type Ca^{2+} current in freshly dissected neurons from dorsal root ganglia (DRG neurons) of adult rats (Todorovic and Lingle (1998) J. Neurophysiol. 79:240–252), and may have limited efficacy in the treatment of abnormal, chronic pain syndromes that follow peripheral nerve damage.

Molecular cloning has revealed the cDNA and corresponding amino acid sequences of several different α1 subunits (α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E}, α_{1G}, α_{1H}, α_{1I}, and α_{1S}). While the cloned α1 subunits identified thus far correspond to several of the calcium channels found in cells, they do not account for all types of calcium conductance found in native cells.

The present invention relates to the discovery of human T-type calcium channel α_{1I} subunit variants that are useful in diagnosis of disease states associated with the peripheral nervous system and for screening compounds that may be used in the treatment of mammals for these disease states.

SUMMARY OF THE INVENTION

The invention is based on the discovery of human T-type calcium channel α_{1I} subunit variants (TCCV-1 and TCCV-2), the polynucleotides encoding TCCV-1 or TCCV-2, and the use of these compositions in screening for compounds effective in treating disease states associated with peripheral pain, and the use of these compositions for diagnosis of these disease states. In particular, the present invention expression vectors, host cells, antibodies, diagnostic kits, and transgenic/knockout animals are provided.

The invention features an isolated polynucleotide encoding TCCV-1 or TCCV-2 polypeptides. The invention further provides an isolated polynucleotide, encoding a TCCV-1 or TCCV-2 polypeptide wherein the polynucleotide encodes an TCCV-1 or TCCV-2 polypeptide comprising the amino acid sequence of SEQ ID NO:2 or 4, respectively. In certain embodiments, the polynucleotide is detectably labeled or is complementary to the polynucleotide encoding a TCCV-1 or TCCV-2 polypeptide. The complementary polynucleotide can also be detectably labeled. In another embodiment, the polynucleotide comprises the nucleic acid sequence of SEQ ID NO:1 or 3.

The present invention encompasses an expression vector comprising the polynucleotide encoding SEQ ID NO:2 or 4. Also contemplated is a host cell comprising the polynucleotide encoding SEQ ID NO:2 or 4. The host cell can be a prokaryotic or eukaryotic cell. The invention further comprises a method of producing a TCCV-1 or TCCV-2 polypeptide comprising: culturing the host cell comprising the expression vector comprising the polynucleotide encoding SEQ ID NO:2 or 4 under conditions suitable for expression of the polypeptide; and recovering the polypeptide from the host cell.

The present invention also contemplates a method of detecting a polynucleotide encoding a TCCV-1 or TCCV-2 polypeptide in a sample containing nucleic acid material, comprising the steps of: contacting the sample with a polynucleotide which is the complement of the polynucleotide encoding SEQ ID NO:2 or 4, wherein the complement is detectably labeled, under conditions suitable for formation of a hybridization complex; and detecting the complex, wherein the presence of the complex is indicative of the presence of the polynucleotide encoding the polypeptide in the sample.

The present invention provides a diagnostic test kit comprising: the polynucleotide comprising SEQ ID NO:1 or 3; and instructions for conducting the diagnostic test.

The present invention encompasses a method of screening for a compound that modulates TCCV-1 or TCCV-2 activity comprising: contacting TCCV-1 or TCCV-2, or fragment thereof with the compound; and detecting modulation of TCCV-1 or TCCV-2 activity. In certain embodiments, the TCCV-1 or TCCV-2 is expressed on a cell or tissue or immobilized on a solid support. The compound can be an antagonist or agonist of TCCV-1 or TCCV-2 activity. In a further embodiment, the compound is ethosuximide or an analog thereof.

The present invention provides an isolated TCCV-1 or TCCV-2 polypeptide or fragment thereof. In certain embodiments, the polypeptide comprises the amino acid sequence of SEQ ID NO:2 or 4. The polypeptide is recombinantly produced or synthetically produced. The present

invention also provides an isolated antibody which specifically binds to the polypeptide of SEQ ID NO:2 or 4.

The present invention encompasses a transgenic nonhuman mammal comprising the polynucleotide encoding TCCV-1 or TCCV-2 polypeptide. The transgenic nonhuman mammal can also comprise the polynucleotide which is the -complement of the polynucleotide encoding TCCV-1 or TCCV-2 which is capable of hybridizing to a polynucleotide encoding TCCV-1 or TCCV-2, thereby reducing expression of TCCV-1 or TCCV-2.

BRIEF DESCRIPTION OF FIGURES AND SEQUENCE IDENTIFIERS

FIGS. 1A–1F show the amino acid alignment between TCCV-1 (SEQ ID NO:2), TCCV-2 (SEQ ID NO:4), and rat T-type Calcium Channel subunit α_{1I} (GenBank Accession No. AAD17796; SEQ ID NO:5). Residues that differ between the rat and human sequences are indicated in bold.

FIGS. 2A–2C show the splicing differences between the 3' ends of TCCV-1 (nucleotides 5148 through 6015 of SEQ ID NO:1) or TCCV-2 (nucleotides 5148 through 6054 of SEQ ID NO:3), and GenBank Accession No. AF086827 (nucleotides 4927 through 5847 of SEQ ID NO:12). Downward pointing arrows indicate exon boundaries. Forward arrows indicate forward PCR primers (Primer Numbers 6352, 6344/88, 6495, and 6495/37). Reverse arrows indicate reverse or antisense PCR primers (Primer Number 6831). Nucleotide differences in the rat sequence which differ from the human PCR primer sequences are underlined.

FIG. 3 shows a 2.0% agarose gel of PCR products following 36 cycles of amplifications using various primers as shown in FIGS. 2A–2C on human brain cDNA. Lane 1 is a 100 bp ladder (Life Technologies, Bethesda, Md.); Lane 3 is the PCR product following amplification with forward primer 6352 and reverse primer 6831; Lane 4 is the PCR product following amplification with forward primer 6344/88 and reverse primer 6831; Lane 5 is the PCR product following amplification with forward primer 6495 and reverse primer 6831; and Lane 6 is the result of amplification with forward primer 6495/37 and reverse primer 6831.

SEQ ID NO:1 is the polynucleotide sequence for TCCV-1. SEQ ID NO:2 is the putative encoded polypeptide.

SEQ ID NO:3 is the polynucleotide sequence for TCCV-2. SEQ ID NO:4 is the putative encoded polypeptide.

SEQ ID NO:5 is the amino acid sequence of GenBank Accession No. AAD17796.

SEQ ID NO:6 through SEQ ID NO:11 are PCR primers used in assembly of full length TCCV-1 and TCCV-2.

SEQ ID NO:12 is the nucleic acid sequence of GenBank Accession No. AF086827.

DETAILED DESCRIPTION OF THE INVENTION

Before the present proteins, nucleotide sequences, and methods are described, it is to be understood that the present invention is not limited to the particular methodologies, protocols, cell lines, vectors, and reagents described, as these may vary. It is also understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not to limit the scope of the present invention.

The singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

All technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention pertains. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of protein chemistry and biochemistry, molecular biology, microbiology and recombinant DNA technology, which are within the skill of the art. Such techniques are explained fully in the literature.

Although any machines, materials, and methods similar or equivalent to those described herein can be used to practice or test the present invention, the preferred machines, materials, and methods are now described. All patents, patent applications, and publications mentioned herein, whether supra or infra, are each incorporated by reference in its entirety.

Definitions

"TCCV" refers to the amino acid sequences of substantially purified TCCV-1 or TCCV-2 obtained from any species particularly mammalian species, including bovine, ovine, porcine, murine, equine, and preferably the human species, from any source, whether natural, synthetic, semi-synthetic, or recombinant.

"Agonist" refers to a molecule which, when bound to TCCV-1 or TCCV-2, or is within proximity of TCCV-1 or TCCV-2, modulates the activity of TCCV-1 or TCCV-2 by increasing or prolonging the duration of the effect of TCCV-1 or TCCV-2. Agonists can include proteins, nucleic acids, carbohydrates, organic compounds, inorganic compounds, or any other molecules which modulate the effect of TCCV-1 or TCCV-2.

An "allelic variant" as used herein, is an alternative form of the gene encoding TCCV-1 or TCCV-2. Allelic variants may result from at least one mutation in the nucleic acid sequence and may result in altered mRNAs or in a polypeptide whose structure or function may or may not be altered. Any given recombinant gene may have none, one, or several allelic forms. Common mutational changes which give rise to allelic variants are generally ascribed to natural deletions, additions, or substitutions of nucleotides. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

"Amplification" relates to the production of additional copies of a nucleic acid sequence. Amplification can be carried out using polymerase chain reaction (PCR) technologies or other methods well known in the art.

The term "analog" is used herein in the conventional pharmaceutical sense. In chemical terminology, an analog refers to a molecule that structurally resembles a referent molecule but which has been modified in a targeted and controlled manner to replace a certain substituent of the referent molecule with an alternate substituent other than hydrogen.

"Antagonist" refers to a molecule which, when bound to TCCV-1 or TCCV-2 or within close proximity, decreases the amount or the duration of the biological or immunological activity of TCCV-1 or TCCV-2. Antagonists may include proteins, nucleic acids, carbohydrates, antibodies, organic compounds, inorganic compounds, or any other molecules which exert an effect on TCCV-1 or TCCV-2 activity.

"Antibody" can be an intact molecule or fragments thereof, such as Fab, $F(ab)_2$, and Fv fragments, which are capable of binding an epitopic determinant. The antibody can be polyclonal, monoclonal, or recombinantly produced.

The terms "antigenic determinant" or "epitopic determinant" refer to the fragment of a molecule that makes contact with a particular antibody.

The term "antisense" refers to any composition containing nucleic acids which is complementary to the "sense" strand of a specific nucleic acid molecule. Antisense molecules

may be produced by any method including synthesis or transcription. Once introduced into a cell, the complementary nucleotides combine with natural sequences produced by the cell to form duplexes and to block either transcription or translation. The designation "negative" can refer to the antisense strand, and the designation "positive" can refer to the sense strand.

A "coding sequence" is a polynucleotide sequence that is transcribed into mRNA and translated into a polypeptide. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to, mRNA, cDNA, synthetic DNA, and recombinant polynucleotide sequences. Also included is genomic DNA where the coding sequence is interrupted by introns.

"Complementary" and "complementarity" refer to the natural binding of polynucleotides to other polynucleotides by base pairing. For example, the sequence "5' A-C-G-T 3'" will bind to the complementary sequence "3' T-G-C-A 5'." Complementarity between two single stranded molecules may be "partial," such that only some of the nucleic acids bind, or it may be "complete," such that total complementarity exists between the single stranded molecules.

A "composition comprising a given polynucleotide sequence" and a "composition comprising a given amino acid sequence" refer broadly to any composition containing the given polynucleotide or amino acid sequence.

The term "control elements" refers collectively to promoters, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell. Not all of these control sequences need always be present in a recombinant vector so long as the desired gene is capable of being transcribed and translated.

The phrase "correlates with expression of a polynucleotide" refers to the detection of the presence of nucleic acids, the same or related to a nucleic acid sequence encoding TCCV-1 or TCCV-2, e.g., by northern analysis or RT-PCR, is indicative of the presence of nucleic acids encoding TCCV-1 or TCCV-2 in a sample, and thereby is indicative of the expression of the transcript from the polynucleotide encoding TCCV-1 or TCCV-2.

The phrase "detectably labeled" as used herein means joining, either covalently or non-covalently to the polynucleotides, polypeptides, or antibodies of the present invention, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are well known in the art. Suitable labels include radionuclides, e.g., ^{32}P, ^{35}S, ^{3}H, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like.

The phrase "disease state" means any disease, condition, symptom, or indication.

The term "expression" as used herein intends both transcriptional and translational processes, i.e., the production of messenger RNA and/or the production of protein therefrom.

The term "hybridization complex" refers to a complex formed between two nucleic acid sequences by virtue of the formation of hydrogen bonds between complementary bases. A hybridization complex may be formed in solution (conditions calculated by performing, e.g., C_0t or R_0t) or formed between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized on a solid support (e.g., paper, membranes, filters, chips, pins, glass slides, or any other appropriate substrate to which cells or their nucleic acids have been fixed.)

An "isolated polynucleotide" that encodes a particular polypeptide refers to a polynucleotide that is substantially free of other nucleic acid molecules that do not encode the subject polypeptide; however, the molecule may include functionally and/or structurally conservative mutations as defined herein.

The term "modulate" refers to a change in the activity of TCCV-1 or TCCV-2. For example, modulation may cause an increase or a decrease in protein activity, binding characteristics, or any other biological, functional, or immunological properties of TCCV-1 or TCCV-2. The ability to modulate the activity of TCCV-1 or TCCV-2 can be exploited in assays to screen for organic, inorganic, or biological compounds which affect the above properties of TCCV-1 or TCCV-2.

"Nucleic acid" and "nucleic acid sequence" refer to a nucleotide, oligonucleotide, polynucleotide, or any fragment thereof. These phrases also refer to DNA or RNA of genomic or synthetic origin which may be single stranded or double stranded and may represent the sense of the antisense strand, a peptide nucleic acid (PNA), or any DNA-like or RNA-like material. In this context, "fragments" refer to those nucleic acids which, when translated, would produce polypeptides retaining some functional characteristic, e.g., antigenicity, or structural domain, e.g., ion channel domain, characteristic of the full-length polypeptide.

The terms "operably associated" and "operably linked" refer to functionally related but heterologous nucleic acid sequences. A promoter is operably associated or operably linked with a coding sequence if the promoter controls the translation or expression of the encoded polypeptide. While operably associated or operably linked nucleic acid sequences can be contiguous and in the same reading frame, certain genetic elements, e.g., repressor genes, are not contiguously linked to the sequence encoding the polypeptide but still bind to operator sequences that control expression of the polypeptide.

An "oligonucleotide" refers to a nucleic acid molecule of at least about 6 to 50 nucleotides, preferably about 15 to 30 nucleotides, and more preferably 20 to 25 nucleotides, which can be used in PCR amplification or in a hybridization assay. "Oligonucleotide" is substantially equivalent to the terms "amplimer," "primer," "oligomer," and "probe" as these terms are commonly defined in the art.

"Peptide nucleic acid" (PNA) refers to an antisense molecule or anti-gene agent which comprises an oligonucleotide of at least 5 nucleotides in length linked to a peptide backbone of amino acid residues ending in lysine. The terminal lysine confers solubility to the composition. PNAs preferentially bind complementary single stranded DNA or RNA and stop transcript elongation, and may be pegylated to extend their lifespan in the cell.

The phrases "percent identity" and "% identity" refers to the percentage of sequence similarity found by a comparison or alignment of two or more amino acid or nucleic acid sequences. Percent identity can be determined by a direct comparison of the sequence information between two molecules by aligning the sequences, counting the exact number of matches between the two aligned sequences, dividing by the length of the shorter sequence, and multiplying the result by 100. Readily available computer programs can be used to aid in the analysis, such as ALIGN, Dayhoff, M. O. in *Atlas of Protein Sequence and Structure* M. O. Dayhoff ed., 5 Suppl. 3:353–358, National Biomedical Research Foundation, Washington, D.C., which adapts the local homology algorithm of Smith and Waterman (1981) *Advances in Appl. Math.* 2:482–489, for peptide analysis.

Programs for determining nucleotide sequence identity are available in the Wisconsin Sequence Analysis Package, Version 8 (Genetics Computer Group, Madison, Wis.) for example, the BLAST, BESTFIT, FASTA, and GAP programs, which also rely on the Smith and Waterman algorithm. These programs are readily utilized with the default parameters recommended by the manufacturer and described in the Wisconsin Sequence Analysis Package referred to above. Other programs for calculating identity or similarity between sequences are known in the art.

"Recombinant host cells," "host cells," "cells," "cell lines," "cell cultures," and other such terms denoting cell lines cultured as unicellular entities refer to cells which can be, or have been, used as recipients for recombinant vectors or other transfer DNA, immaterial of the method by which the DNA is introduced into the cell or the subsequent disposition of the cell. The terms include the progeny of the original cell which has been transfected. Cells in primary culture as well as cells such as oocytes also can be used as recipients.

A "reporter gene" is a gene that, upon expression, confers a phenotype on a cell expressing the reporter gene, such that the cell can be identified under appropriate conditions. For example, the reporter gene may produce a polypeptide product that can be easily detected or measured in a routine assay. Suitable reporter genes known in the art which confer this characteristic include those that encode chloramphenicol acetyl transferase (CAT activity), β-galactosidase, luciferase, alkaline phosphatase, human growth hormone, fluorescent proteins, such as green fluorescent protein (GFP), and others. Indeed, any gene that encodes a protein or enzyme that can readily be measured, for example, by an immunoassay such as an enzyme-linked immunosorbent assay (ELISA) or by the enzymatic conversion of a substrate into a detectable product, and that is substantially not expressed in the host cells (specific expression with no background) can be used as a reporter gene to test for promoter activity. Other reporter genes for use herein include genes that allow selection of cells based on their ability to thrive in the presence or absence of a chemical or other agent that inhibits an essential cell function. Suitable markers, therefore, include genes coding for proteins which confer drug resistance or sensitivity thereto, or change the antigenic characteristics of those cells expressing the reporter gene when the cells are grown in an appropriate selective medium. For example, reporter genes include: cytotoxic and drug resistance markers, whereby cells are selected by their ability to grow on media containing one or more of the cytotoxins or drugs; auxotrophic markers by which cells are selected by their ability to grow on defined media with or without particular nutrients or supplements; and metabolic markers by which cells are selected for, e.g., their ability to grow on defined media containing the appropriate sugar as the sole carbon source. These and other reporter genes are well known in the art.

A "change in the level of reporter gene product" is shown by comparing expression levels of the reporter gene product in a cell exposed to a candidate compound relative to the levels of reporter gene product expressed in a cell that is not exposed to the test compound and/or to a cell that is exposed to a control compound. The change in level can be determined quantitatively for example, by measurement using a spectrophotometer, spectrofluorometer, luminometer, and the like, and will generally represent a statistically significant increase or decrease in the level from background. However, such a change may also be noted without quantitative measurement simply by, e.g., visualization, such as when the reporter gene is one that confers the ability on cells to form colored colonies on chromogenic substrates.

The term "sample" is used in its broadest sense. A sample suspected of containing nucleic acids encoding TCCV-1 or TCCV-2, or fragments thereof, or TCCV-1 or TCCV-2 polypeptide may comprise a bodily fluid; an extract from a cell chromosome, organelle, or membrane isolated from a cell; an intact cell; genomic DNA, RNA, or cDNA, in solution or bound to a substrate; a tissue; a tissue print; etc.

"Stringent conditions" refers to conditions which permit hybridization between polynucleotides and the claimed polynucleotides. Stringent conditions can be defined by salt concentration, the concentration of organic solvent, e.g., formamide, temperature, and other conditions well known in the art.

"Subject" means mammals and non-mammals. Mammals means any member of the Mammalia class including, but not limited to, humans, non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, horses, sheep, goats, and swine; domestic animals such as rabbits, dogs, and cats; laboratory animals including rodents, such as rats, mice, and guinea pigs; and the like. Examples of non-mammals include, but are not limited to, birds, and the like. The term "subject" does not denote a particular age or sex.

The term "substantially purified," when referring to a polypeptide, indicates that the polypeptide is present in the substantial absence of other similar biological macromolecules.

The term "transfection" refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion, or the molecular form of the polynucleotide that is inserted. The insertion of a polynucleotide per se and the insertion of a plasmid or vector comprised of the exogenous polynucleotide are included. The exogenous polynucleotide may be directly transcribed and translated by the cell, maintained as a nonintegrated vector, for example, a plasmid, or alternatively, may be stably integrated into the host genome.

The term "transformed" refers to any known method for the insertion of foreign DNA or RNA sequences into a host prokaryotic cell. The term "transfected" refers to any known method for the insertion of foreign DNA or RNA sequences into a host eukaryotic cell. Such transformed or transfected cells include stably transformed or transfected cells in which the inserted DNA is rendered capable of replication in the host cell. They also include transiently expressing cells which express the inserted DNA or RNA for limited periods of time. The transformation or transfection procedure depends on the host cell being transformed. It can include packaging the polynucleotide in a virus as well as direct uptake of the polynucleotide, such as, for example, lipofection or microinjection. Transformation and transfection can result in incorporation of the inserted DNA into the genome of the host cell or the maintenance of the inserted DNA within the host cell in plasmid form. Methods of transformation are well known in the art and include, but are not limited to, viral infection, electroporation, lipofection, and calcium phosphate mediated direct uptake. "Treating" or "treatment" of a disease state includes: 1) preventing the disease state, i.e. causing the clinical symptoms of the disease state not to develop in a subject that may be exposed to or predisposed to the disease state, but does not yet experience or display symptoms of the disease state; 2) inhibiting the disease state, i.e., arresting the development of the disease state or its clinical symptoms; 3) or relieving the disease state, i.e., causing temporary or permanent regression of the disease state or its clinical symptoms.

A "variant" of TCCV-1 or TCCV-2 polypeptides refers to an amino acid sequence that is altered by one or more amino acid residues. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties (e.g., replacement of leucine with isoleucine.) More rarely, a variant may have "nonconservative" changes (e.g., replacement of glycine with tryptophan.) Analogous minor variations may also include amino acid deletion or insertions, or both. Guidance in determining which amino acid variations may be substituted, inserted, or deleted without abolishing biological function may be found using programs well known in the art, for example, LASERGENE software (DNASTAR).

The term "variant" when used in the context of a polynucleotide sequence, may encompass a polynucleotide sequence related to TCCV-1 or TCCV-2. This definition may include, for example "allelic" (as defined above), "splice," "species," "polymorphic," or "degenerate" variants. A splice variant may have significant identity to a reference molecule, but will generally have a greater of less number polynucleotides due to alternate splicing of exons during mRNA processing. The corresponding polypeptide may possess additional functional domains or an absence of domains. Species variants are polynucleotide sequences that vary from one species to another. The resulting polypeptides generally will have significant amino acid identity to each other. A polymorphic variant is a variation in the polynucleotide sequence of a particular gene between individuals within a given species. Polymorphic variants may also encompass "single nucleotide polymorphisms" (SNPs) in which the polynucleotide sequence varies by one base. The presence of SNPs may be indicative of, for example, a certain population, a disease state, or a propensity for a disease state. A degenerate variant encompasses a multitude of polynucleotides which encode TCCV-1 or TCCV-2 polypeptides. The degenerate variants may occur naturally or may be produced synthetically. Synthetic degenerate variants are made in accordance with the standard triplet genetic code as applied to the polynucleotide sequence of naturally occurring TCCV-1 or TCCV-2, and all such variations are to be considered as being specifically disclosed.

A "vector" is a replicon in which another polynucleotide segment is attached, such as to bring about the replication and/or expression of the attached segment. The term includes expression vectors, cloning vectors, and the like.

The Invention

The present invention is based on the discovery of a human T-type calcium channel α_{1I} subunit variant (TCCV-1 or TCCV-2), the polynucleotides encoding TCCV-1 or TCCV-2, and the use of these compositions for screening compounds useful in the treatment or prevention of pain, including, but not limited to peripheral pain; peripheral neuropathies; pain caused by trauma or toxic compounds; diabetic neuropathy; cancer pain, and the like.

The molecules of the present invention were isolated by homology searching of the GenBank database using the rat T-type calcium channel Otl G subunit (see, e.g., Perez-Reyes et al. (1 998) *Nature* 391:896–900; and GenBank Accession No. AF027984) and the human α1H subunit. (See, e.g., Cribbs et al. (1998) Circ. Res. 83:103–109; and GenBank Accession No. AF051946.) Two genomic clones (GenBank Accession No. AL02231 9 and AL00871 6) from human chromosome 22 were identified as being homologous to the two subunits.

Through PCR extension and use of sequence analysis software, TCCV-1 and TCCV-2 were assembled. TCCV-1 is a 6816 bp polynucleotide (SEQ ID NO:l) encoding a polypeptide of 2175 amino acid residues (SEQ ID NO:2). TCCV-2 is a 6855 bp polynucleotide (SEQ ID NO:3) encoding a polypeptide of 2188 amino acid residues (SEQ ID NO:4). FIGS. 1A–1F show an amino acid alignment between TCCV-1. TCCV-2, and the rat $\alpha 1_I$ subunit (GenBank Accession No. AAD17796; SEQ ID NO:5). The overall sequence identity between TCCV-1 and AAD17796 is approximately 77%, with 93% identity from residues 1 through 1823 of SEQ ID NO:2. A unique fragment of SEQ ID NO:2 from about residuel 1811 through about residue 2175 is useful, e.g., as an immungenic polypeptide. The corresponding polynucleotide sequence from about nucleotide 5622 through about nucleotide 6716 of SEQ ID NO:1 is useful, e.g., as a hybridization probe. A unique fragment of SEQ ID NO:4 from about residue 1824 through about residue 2188 is useful, e.g., as an immunogenic polypeptide. The corresponding polynucleotide fragment from about nucleotide 5661 through about nucleotide 6755 is useful, e.g., as a hybridization probe.

PCR analysis was performed using forward primers spanning exons 31 and 32 of SEQ ID NO:1, 3, and 12 (Primer Number 6352 for SEQ ID NO:1 and 12, and Primer Number 6344/88 for SEQ ID NO:3) and exons 32 and 33 of SEQ ID NO:1, 3, and 12 (Primer Number 6495 for SEQ ID NO:1 and 3, and Primer Number 6495/37 for SEQ ID NO:12) in combination with a reverse primer (Primer Number 6831 for SEQ ID NO: 1, 3, and 12). The results are illustrated in FIG. 3. No PCR product was detected using forward Primer Number 6493/37 and reverse Primer Number 6831 (lane 6).

The invention also encompasses nucleic or amino acid variants of TCCV-1 or TCCV-2. A preferred variant is one which has at least about 80%, more preferably at least about 90%, and most preferably at least about 95% amino acid or nucleic acid identity to the corresponding TCCV-1 or TCCV-2 sequence, and which contains at least one functional or structural characteristic of TCCV-1 or TCCV-2.

Polynucleotides

Although nucleotide sequences which encode TCCV-1 or TCCV-2 and its variants are preferably capable of hybridizing to the nucleotide sequence of the naturally occurring TCCV-1 or TCCV-2 under appropriately selected conditions of stringency, it may be advantageous to produce nucleotide sequence encoding TCCV-1 or TCCV-2 or its derivatives possessing a substantially different codon usage, e.g., inclusion of non-naturally occurring codons. Codons may be selected to increase the rate at which expression of the peptide occurs in a particular prokaryotic or eukaryotic host in accordance with the frequency with which particular codons are utilized by the host. Other reasons for substantially altering the nucleotide sequence encoding TCCV-1 or TCCV-2 and its derivatives without altering the encoded amino acid include the production of RNA transcripts having more desirable properties, such as greater half-lifer or stability for improved translation, than transcripts produced from the naturally occurring sequence.

Also encompassed by the invention are polynucleotides that are capable of hybridizing to the claimed polynucleotide sequences, and, in particular, to those shown in SEQ ID NOs:1 and 3, under various conditions of stringency. (See, e.g., Wahl, G. M. and S. L. Berger (1987) Methods Enzymol. 152:399–401; Kimmel, A. R. (1987) Methods Enzymol. 152:507–511.) For example, stringent salt concentration will ordinarily be less that about 750 mM NaCl and 75 mM trisodium citrate, preferably less than about 500 mM NaCl and 50 mM trisodium citrate, and more preferably less than about 250 mM NaCl and 25 mM trisodium citrate. Low stringency hybridization can be obtained in the absence of

organic solvent, e.g., formamide, while high stringency hybridization can be obtained in the presence of at least about 35% formamide, and more preferably at least about 50% formamide. Stringent temperature conditions will ordinarily include temperatures of at least about 30° C., preferably at least about 37° C., and more preferably 42° C. Varying additional parameters such as hybridization time, the concentration of detergent, e.g., sodium dodecyl sulfate (SDS), and the inclusion or exclusion of carrier DNA, are well known to those skilled in the art. Various levels of stringency are accomplished by combining these various conditions as needed. In a preferred embodiment, hybridization will occur at 30° C. in 750 mM NaCl, 75 mM trisodium citrate, and 1% SDS. In a more preferred embodiment, hybridization will occur at 37° C. in 500 mM NaCl, 50 mM trisodium citrate, 1% SDS, 35% formamide, and 100 μg/ml denatured salmon sperm DNA (ssDNA). In a more preferred embodiment, hybridization will occur at 42° C. in 250 mM NaCl, 25 mM trisodium citrate, 1% SDS, 50% formamide, and 200 μg/ml denatured ssDNA. Useful variations of these conditions will be readily apparent to those skilled in the art.

The washing steps which follow hybridization can also vary in stringency. Wash stringency conditions can be defined by salt concentration and by temperature. As above, wash stringency can be increased by decreasing salt concentration or by increasing temperature. For example, stringent salt concentration for the wash steps will preferably be less than about 30 mM NaCl and 3 mM trisodium citrate, and most preferably less than about 15 mM NaCl and 1.5 mM trisodium citrate. Stringent temperature conditions for the wash step will ordinarily include temperature of at least about 25° C., more preferably of at least about 42° C., and most preferably of at least about 68° C. In a preferred embodiment, wash step will occur at 25° C. in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash step will occur at 42° C. in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. In a most preferred embodiment, the wash step will occur at 68° C., in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. Additional variations on these conditions will be readily apparent to those skilled in the art.

In another embodiment, polynucleotide sequences encoding all or part of TCCV-1 or TCCV-2 may be synthesized using chemical methods well known in the art. (See, e.g., Caruthers, M. H. et al. (1980) Nucl. Acids Res. Symp. Ser. 7:215–223, Hom, T. et al. (1980) Nucl. Acids Res. Symp. Ser. 7:225–232.)

The present invention further covers recombinant polynucleotides and fragments having a DNA sequence identical to or highly homologous to the isolated polynucleotides of TCCV-1 or TCCV-2. In particular, the sequences will often be operably linked to DNA segments which control transcription, translation, and DNA replication. Alternatively, recombinant clones derived from the genomic sequences, e.g., containing introns, will be useful for transgenic and knock-out studies, including transgenic cells, organisms, and knock-out animals, and for gene therapy. (See, e.g., Goodnow (1992) "Transgenic Animals" in Roitt (ed.) Encyclopedia of Immunology, Academic Press, San Diego, Calif., pp.1502–1504; Travis (1992) Science 254:707–710; Capecchi (1989) Science 244:1288–1292; Robertson (ed.) (1987) Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, IRL Press, Oxford; Rosenberg (1992) J. Clinical Oncology 10:180–199; Hogan, et al. (eds.) (1994) Manipulating the Mouse Embryo: A Laboratory Manual, 2nd edition, Cold Spring Harbor Press, N.Y.; Wei (1997) Ann. Rev. Pharmacol. Toxicol. 37:119–141; and Rajewsky, et al. (1996) J. Clin. Inves. 98:S51–S53.)

Examples of these techniques include: 1) Insertion of normal or mutant versions of DNA encoding TCCV-1 or TCCV-2 or homologous animal versions of these genes, by microinjection, retroviral infection, or other means well known to those skilled in the art, into appropriate fertilized embryos in order to produce a transgenic animal (see, e.g., Hogan, supra); and 2) homologous recombination (see, e.g., Capecchi, supra; and Zimmer and Gruss (1989) Nature 338:150–153) of mutant or normal, human or animal versions of these genes with the native gene locus in transgenic animals to alter the regulation of expression or the structure of TCCV-1 or TCCV-2.

The technique of homologous recombination is well known in the art. It replaces the native gene with the inserted gene and is thus useful for producing an animal that cannot express native receptor but does express, for example, an inserted mutant receptor, which has replaced the native receptor in the animal's genome by recombination, resulting in underexpression of the receptor.

Microinjection adds genes to the genome, but does not remove them, and so is useful for producing an animal which expresses its own and added receptors, resulting in overexpression of the receptor. One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium (see, e.g., Hogan, supra). DNA or cDNA encoding TCCV-1 or TCCV-2 is purified from an appropriate vector by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the trans-gene. Alternatively, or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the trans-gene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipet puller) and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for inserting DNA into the egg cell, and is used here only for exemplary purposes.

Since the normal action of receptor-specific drugs is to activate or to inhibit the receptor, the transgenic animal model systems described above are useful for testing the biological activity of drugs directed against TCCV-1 or TCCV-2 even before such drugs become available. These animal model systems are useful for predicting or evaluating possible therapeutic applications of drugs which activate or inhibit TCCV-1 or TCCV-2 by inducing or inhibiting expression of the native or trans-gene and thus increasing or decreasing expression of normal or mutant TCCV-1 or TCCV-2 in the living animal. Thus, a model system is produced in which the biological activity of drugs directed against TCCV-1 or TCCV-2 are evaluated before such drugs become available.

The transgenic animals which over- or underproduce TCCV-1 or TCCV-2 indicate, by their physiological state, whether over- or underproduction of TCCV-1 or TCCV-2 is

therapeutically useful. It is therefore useful to evaluate drug action based on the transgenic model system. One use is based on the fact that it is well known in the art that a drug such as an antidepressant acts by blocking neurotransmitter uptake, and thereby increases the amount of neurotransmitter in the synaptic cleft. The physiological result of this action is to stimulate the production of less receptor by the affected cells, leading eventually to underexpression. Therefore, an animal which underexpresses receptor is useful as a test system to investigate whether the actions of such drugs which result in under expression are in fact therapeutic. Another use is that if overexpression is found to lead to abnormalities, then a drug which down-regulates or acts as an antagonist to TCCV-1 or TCCV-2 is indicated as worth developing, and if a promising therapeutic application is uncovered by these animal model systems, activation or inhibition of TCCV-1 or TCCV-2 is achieved therapeutically either by producing agonist or antagonist drugs directed against TCCV-1 or TCCV-2 or by any method which increases or decreases the expression of TCCV-1 or TCCV-2 in man.

Polypeptides

The predicted sequence of TCCV-1 and TCCV-2 amino acid sequence is shown in SEQ ID NO:2 and SEQ ID NO:4, respectively. The peptide sequences allow preparation of peptides to generate antibodies to recognize such segments, and various different methods may be used to prepare such peptides. As used herein TCCV-1 or TCCV-2 shall encompass, when used in a protein context, a protein having an amino acid sequence shown in Table 2, or a significant fragment of such a protein. It also refers to a vertebrate, e.g., mammal, including human, derived polypeptide which exhibits similar biological function, e.g., antigenic, or interacts with TCCV-1 or TCCV-2 specific binding components, e.g., specific antibodies.

The term polypeptide, as used herein, includes a significant fragment or segment, and encompasses a stretch of amino acid residues of at least about 8 amino acids, generally at least 10 amino acids, more generally at least 12 amino acids, often at least 14 amino acids, more often at least 16 amino acids, typically at least 18 amino acids, more typically at least 20 amino acids, usually at least 22 amino acids, more usually at least 24 amino acids, preferably at least 26 amino acids, more preferably at least 28 amino acids, and, in particularly preferred embodiments, at least about 30 or more amino acids. The segments may have lengths of at least 37, 45, 53, 61, 70, 80, 90, etc., and often will encompass a plurality of such matching sequences. The specific ends of such a segment will be at any combinations within the protein. Preferably the fragment will encompass structural domains, e.g., [Give specific fragments], or unique regions useful in generation of binding compositions with specificity for TCCV-1 or TCCV-2.

In another embodiment of the invention, natural, modified, or recombinant nucleic acid sequences encoding TCCV-1 or TCCV-2 may be ligated to a heterologous sequence to encode a fusion protein. For example, to screen peptide libraries for inhibitors of TCCV-1 or TCCV-2 activity, it may be useful to encode a chimeric TCCV-1 or TCCV-2 protein that can be recognized by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between the TCCV-1 or TCCV-2 encoding sequence and the heterologous protein sequence, so that TCCV-1 or TCCV-2 may be cleaved and purified away from the heterologous moiety.

The protein may be produced using chemical methods to synthesize the amino acid sequence of TCCV-1 or TCCV-2, or a fragment thereof. For example, peptide synthesis can be performed using various solid-phase techniques. (See, e.g., Roberge, J. Y. et al. (1995) Science 269:202–204.) Automated synthesis may be achieved, for example, using the ABI 431 A peptide synthesizer (Perkin Elmer).

The newly synthesized peptide may be substantially purified by preparative high performance liquid chromatography. (See, e.g., Creighton, T. (1983) Proteins, Structures and Molecular Principles, WH Freeman and Co., New York, N.Y.) The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; Creighton, supra.) Additionally, the amino acid sequence of TCCV-1 or TCCV-2, or any part thereof, may be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins, or any part thereof, to produce a variant polypeptide.

In order to express a biologically active TCCV-1 or TCCV-2, the nucleotide sequences encoding TCCV-1 or TCCV-2 or functional equivalents, may be inserted into appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. Methods which are well known to those skilled in the art may be used to construct expression vectors containing sequences encoding TCCV-1 or TCCV-2 and appropriate transcriptional and translational control elements. These methods include in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. (See, e.g., Sambrook, J. et al. (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y.; and Ausubel, F. M. et al. (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y.)

A variety of expression vector/host systems may be utilized to contain and express sequences encoding TCCV-1 or TCCV-2. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (e.g., baculovirus) or with bacterial expression vectors (e.g., Ti or pBR322 plasmids); or animal cell systems. The invention is not limited by the host cell employed.

The "control elements" or "regulatory sequences" are those non-translated regions of the vector, e.g., enhancers, promoters, 5' and 3' untranslated regions, which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the BLUESCRIPT phagemid (Stratagene, LaJolla, Calif.) or PSPORT1 plasmid (Gibco BRL) and the like may be used. The baculovirus polyhedrin promoter may be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (e.g., heat shock, RUBISCO; and storage protein genes) or from plant viruses (e.g., viral promoters or leader sequences) may be cloned into the vector. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferable. If it is necessary to generate a cell line that contains multiple copies of the sequence encoding TCCV-1 or TCCV-2, vectors based on SV40 or EBV may be used with an appropriate selectable marker.

In bacterial systems, a number of expression vectors may be selected depending upon the use intended for TCCV-1 or

TCCV-2. For example, when large quantities of TCCV-1 or TCCV-2 are needed for the induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified may be used. Such vectors include, but are not limited to, the multifunctional *E. coli* cloning and expression vectors such as the BLUESCRIPT phagemid (Stratagene), in which the sequence encoding TCCV-1 or TCCV-2 may be ligated into the vector in frame with sequences for the amino-terminal Met and the subsequent 7 residues of β-galactosidase so that a hybrid protein is produced; pIN vectors (Van Heeke, G. and S. M. Schuster (1989) J. Biol. Chem. 264:5503–5509); and the like. PGEX vectors (Promega, Madison, Wis.) may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems may be designed to include heparin, thrombin, or factor XA protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will.

In the yeast, *Saccharomyces cerevisiae*, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase, and PGH may be used. (See, e.g., Ausubel et al., supra; and Grant et al. (1987) Methods Enzymol. 153:516–544.)

An insect system may also be used to express TCCV-1 or TCCV-2. For example, in one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia larvae*. The sequences encoding TCCV-1 or TCCV-2 may be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of TCCV-1 or TCCV-2 will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. the recombinant viruses may then be used to infect, for example, *S. frugiperda* cells or *Trichoplusia larvae* in which TCCV-1 or TCCV-2 may be expressed (Engelhard, E. K. et al. (1994) Proc. Nat. Acad. Sci. 91:3224–3227).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, sequences encoding TCCV-1 or TCCV-2 may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome may be used to obtain a viable virus which is capable of expressing TCCV-1 or TCCV-2 in infected host cells (Logan, J. and Shenk, T. (1984) Proc. Natl. Acad. Sci. 81:3655–3659). In addition, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

Human artificial chromosomes (HACs) may also be employed to deliver larger fragments of DNA than can be contained and expressed in a plasmid. HACs of 6 to 10M are constructed and delivered via conventional delivery methods (liposomes, polycationic amino polymers, or vesicles) for therapeutic purposes.

Specific initiation signals may also be used to achieve more efficient translation of sequences encoding TCCV-1 or TCCV-2. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding TCCV-1 or TCCV-2, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a fragment thereof, is inserted, exogenous translational control signals including the ATG initiation codon should be provided. Furthermore, the initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers which are appropriate for the particular cell system which is used, such as those described in the literature (Scharf, D. et al. (1994) Results Probl. Cell Differ. 20:125–162).

In addition, a host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the protein may also be used to facilitate correct insertion, folding and/or function. Different host cells which have specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO, HeLa, MDCK, HEK293, and WI38), are available from the American Type Culture Collection (ATCC; Bethesda, Md.) and may be chosen to ensure the correct modification and processing of the foreign protein.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines, which stably express TCCV-1 or TCCV-2, may be transformed using expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells may be allowed to grow for 1–2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clones of stably transformed cells may be proliferated using tissue culture techniques appropriate to the cell type.

Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler, M. et al. (1977) Cell 11:223–32) and adenine phosphoribosyltransferase (Lowy, I. et al. (1980) Cell 22:817–23) genes which can be employed in tk^- or $aprt^-$ cells, respectively. Also, antimetabolite, antibiotic or herbicide resistance can be used as the basis for selection; for example, dhfr which confers resistance to methotrexate (Wigler, M. et al. (1980) Proc. Natl. Acad. Sci. 77:3567–70); npt, which confers resistance to the aminoglycosides neomycin and G-418 (Colbere-Garapin, F. et al (1981) J. Mol. Biol. 150:1–14). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan, or hisD, which allows cells to utilize histinol in place of histidine (Hartman, S. C. and R. C. Mulligan (1988) Proc. Natl. Acad. Sci. 85:8047–51). Recently, the use of visible markers has gained popularity with such markers as anthocyanins, .beta. glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes, Calif. et al. (1995) Methods Mol. Biol. 55:121–131).

Antibodies

Antibodies to TCCV-1 or TCCV-2 may be generated using methods that are well known in the art. Such antibod-

ies may include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, and fragments produced by a Fab expression library. Neutralizing antibodies, (i.e., those which inhibit dimer formation) are especially preferred for therapeutic use.

For the production of antibodies, various hosts including goats, rabbits, rats, mice, humans, and others, may be immunized by injection with TCCV-1 or TCCV-2 or any fragment or oligopeptide thereof which has immunogenic properties. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. Among adjuvants used in humans, BCG (bacilli Calmette-Guerin) and Corynebacterium parvum are especially preferable.

It is preferred that the oligopeptides, peptides, or fragments used to induce antibodies to TCCV-1 or TCCV-2 have an amino acid sequence consisting of at least five amino acids and more preferably at least 10 amino acids, and most preferably at least 15 amino acids. It is also preferable that they are identical to a portion of the amino acid sequence of the natural protein, and they may contain the entire amino acid sequence of a small, naturally occurring molecule. Short stretches of TCCV-1 or TCCV-2 amino acids may be fused with those of another protein such as keyhole limpet hemocyanin and antibody produced against the chimeric molecule.

Monoclonal antibodies to TCCV-1 or TCCV-2 may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique (Kohler, G. et al. (1975) Nature 256:495–497; Kozbor, D. et al. (1985) J. Immunol. Methods 81:31–42; Cote, R. J. et al. (1983) Proc. Natl. Acad. Sci. 80:2026–2030; Cole, S. P. et al. (1984) Mol. Cell Biol. 62:109–120).

In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used (Morrison, S. L. et al. (1984) Proc. Natl. Acad. Sci. 81:6851–6855; Neuberger, M. S. et al. (1984) Nature 312:604–608; Takeda, S. et al. (1985) Nature 314:452–454). Alternatively, techniques described for the production of single chain antibodies may be adapted, using methods known in the art, to produce TCCV-1 or TCCV-2-specific single chain antibodies. Antibodies with related specificity, but of distinct idiotypic composition, may be generated by chain shuffling from random combinatorial immunoglobulin libraries (Burton D. R. (1991) Proc. Natl. Acad. Sci. 88:11120–3).

Antibodies may also be produced by inducing in vivo production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature (Orlandi, R. et al. (1989) Proc. Natl. Acad. Sci. 86: 3833–3837; Winter, G. et al. (1991) Nature 349:293–299).

Antibody fragments which contain specific binding sites for TCCV-1 or TCCV-2 may also be generated. For example, such fragments include, but are not limited to, the F(ab')2 fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')2 fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse, W. D. et al. (1989) Science 254:1275–1281).

Various immunoassays may be used for screening to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the measurement of complex formation between TCCV-1 or TCCV-2 and its specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering TCCV-1 or TCCV-2 epitopes is preferred, but a competitive binding assay may also be employed (Maddox, supra).

Uses

The present invention provides various methods for determining whether a compound can modulate the activity of TCCV-1 or TCCV-2. The compound can be a substantially pure compound of synthetic origin combined in an aqueous medium, or the compound can be a naturally occurring material such that the assay medium is an extract of biological origin, such as, for example, a plant, animal, or microbial cell extract. The methods essentially entail contacting TCCV-1 or TCCV-2 or fragments thereof, with the compound under suitable conditions and subsequently determining if the compound modulates the activity of TCCV-1 or TCCV-2. The compounds of interest can function as agonists or antagonists of TCCV-1 or TCCV-2 activity. TCCV-1 or TCCV-2 or fragments thereof, can be expressed on a cell or tissue, naturally or recombinantly, or immobilized by attachment to a solid substrate, e.g., nitrocellulose or nylon membrane, glass, beads, etc. An example of a compound that may block a T-type calcium channel is ethosuximide and analogs thereof.

Transcription based assays that identify signals that modulate the activity of cell surface proteins, e.g., receptors, ion channels, etc., may be used to screen candidate compounds for their ability to stimulate reporter gene product expression and their potential to stimulate the expression of TCCV-1 or TCCV-2.

One method for identifying compounds that stimulate TCCV-1 or TCCV-2 promoter-controlled reporter gene expression comprises introducing into a cell a DNA construct that comprises TCCV-1 or TCCV-2 promoter operably linked to a reporter gene, mixing a test compound with the cell and measuring the level of expression of reporter gene product. A change in the level of expression of the reporter gene produced indicates that the compound is capable of modulating the level of TCCV-1 or TCCV-2 expression. The reporter gene construct is preferably stably integrated into the chromosomal DNA of the cell, but is also functional for the purposes disclosed herein in the form of an extrachromosomal element. The cell may be a eukaryotic cell, or any cell that contains the elements needed to express a structural gene under the regulatory influence of a mammalian gene promoter.

Other transcription-based assays are well known in the art. (See, e.g., Zlokamik, et al. (1998) Science 279:84–88; Siverman, supra; and Gonzalez and Negulescu, (1998) Curr. Opin. Biotechnol. 9:624–631.) These transcription based assays asses the intracellular transduction of an extracellular signal using recombinant cells that are modified by introduction of a reporter gene under the control of a regulatable promoter.

A two-hybrid system-based approach can also be employed for compound screening, small molecule

identification, and drug discovery. The underlying premise of the two-hybrid system, originally described in yeast by Fields and Song (1989) Nature 340:245–246, provides a connection between a productive protein-protein or protein-compound interaction pair of interest and a measurable phenotypic change in yeast. A reporter cassette containing an up-stream activation sequence which is recognized by a DNA binding domain, is operationally linked to a reporter gene, which when expressed under the correct conditions will generate a phenotypic change. The original two-hybrid system has recently been modified for applicability in high-throughput compound screening. (See, e.g., Ho et al. (1996) Nature 382:822–826; Licitra and Liu (1996) Proc. Natl. Acad. Sci. USA 93:12817–12821; and Young et al. (1998) Nature Biotech. 16:946–950.)

Assays for identifying compounds that modulate ion channel activity are practiced by measuring the ion channel activity when a cell expressing the ion channel of interest, or fragments thereof, is exposed to a solution containing the test compound and a ion channel selective ion and comparing the measured ion channel activity to the native ion channel activity of the same cell or a substantially identical control cell in a solution not containing the test compound. Methods for practicing such assays are known to those of skill in the art. (See, e.g., Mishina et al. (1985) Nature 313:364–369; and Noda, et al. Nature 322:836–828.)

Ion channel activity can be measured by methods such as electrophysiology (two electrode voltage clamp or single electrode whole cell patch clamp), guanidinium ion flux assays, toxin-binding assays, and Fluorometric Imaging Plate Reader (FLIPR) assays. (See, e.g., Sullivan, et al. (1999) Methods Mol. Biol. 114:125–133; Siegel and Isacoff (1997) Neuron 19:1–20; and Lopatin, et al. (1998) Trends Pharmacol. Sci. 19:395–398.) An "inhibitor" is defined generally as a compound, at a given concentration, that results in greater than 50% decrease in ion channel activity, preferably greater than 70% decrease in ion channel activity, more preferably greater than 90% decrease in ion channel activity.

The binding or interaction of the compound with a receptor or fragments thereof, can be measured directly by using radioactively labeled compound of interest (see, e.g., Wainscott et al. (1993) Mol. Pharmacol. 43:419–426; and Loric, et al. (1992) FEBS Left. 312:203–207) or by the second messenger effect resulting from the interaction or binding of the candidate compound. (See, e.g., Lazereno and Birdsall (1993) Br. J. Pharmacol.109:1120–1127.) Modulation in receptor signaling can be measured using a detectable assay, e.g., the FLIPR assay. (See, e.g., Coward, P. (1999) Anal. Biochem. 270:242–248; Sittampalam, supra; and Gonzalez and Negulescu, supra.) Activation of certain receptors, in particular, GPCRs, can be measured an ^{35}S-GTPγS binding assay. (See, e.g., Lazareno (1999) Methods Mol. Biol. 106:231–245.)

Alternatively, the candidate compounds can be subjected to competition screening assays, in which a known ligand, preferably labeled with an analytically detectable reagent, most preferably radioactivity, is introduced with the drug to be tested and the capacity of the compound to inhibit or enhance the binding of the labeled ligand is measured. Compounds are screened for their increased affinity and selectivity for the specific receptor or fragments thereof.

Candidate compounds are useful in the treatment or prophylaxis of pain, including, but not limited to, peripheral pain; peripheral neuropathies; pain caused by trauma or toxic compounds; diabetic neuropathy; cancer pain, and the like.

The polynucleotides of the present invention can be used to design antisense oligonucleotides that inhibit translation of mRNA encoding the TCCV-1 or TCCV-2 of the present invention. Synthetic oligonucleotides, or other antisense chemical structures are designed to bind to mRNA encoding TCCV-1 or TCCV-2 and inhibit translation of mRNA and are useful to inhibit expression of TCCV-1 or TCCV-2. This invention provides a means to alter levels of expression of TCCV-1 or TCCV-2 by the use of a synthetic antisense oligonucleotide (SAO) which inhibits translation of mRNA encoding these receptors.

The SAO is designed to be capable of passing through cell membranes in order to enter the cytoplasm of the cell by virtue of physical and chemical properties of the SAO which render it capable of passing through cell membranes (e.g. by designing small, hydrophobic SAO chemical structures) or by virtue of specific transport systems in the cell which recognize and transport the SAO into the cell. In addition, the SAO can be designed for administration only to certain selected cell populations by targeting the SAO to be recognized by specific cellular uptake mechanisms which binds and takes up the SAO only within certain selected cell populations. For example, the SAO may be designed to bind to TCCV-1 or TCCV-2 which are found only in certain cell types.

The SAO is also designed to recognize and selectively bind to the target mRNA sequence, which may correspond to a sequence contained within the sequences of SEQ ID NO:1 or 3 by virtue of complementary base pairing to the mRNA. Finally, the SAO is designed to inactivate the target mRNA sequence by any of three mechanisms: 1) binding to the target mRNA and thus inducing degradation of the mRNA by intrinsic cellular mechanisms such as RNAse H digestion; 2) inhibiting translation of the mRNA target by interfering with the binding of translation-regulating factors or of ribosomes; or 3) inclusion of other chemical structures, such as ribozyme sequences or reactive chemical groups, which either degrade or chemically modify the target mRNA.

Synthetic antisense oligonucleotide drugs have been shown to be capable of the properties described above when directed against mRNA targets. (See, e.g., Cohen (1989) Trends in Pharm. Sci. 10:435; and Weintraub (1990) Sci. Am. 262:40–46.) In addition, coupling of ribozymes to antisense oligonucleotides is a promising strategy for inactivating target mRNA. (See, e.g., Sarver et al. (1990) Science 247:1222.)

Diagnostics and kits

The present invention contemplates use TCCV-1 or TCCV-2 polynucleotides, polypeptides, and antibodies in a variety of diagnostic methods kits. Typically the kit will have a compartment containing either a defined TCCV-1 or TCCV-2 polypeptide, polynucleotide, or a reagent which recognizes one or the other, e.g., antigen fragments or antibodies. Additionally the kit will include the reagents needed to carry out the assay in a separate compartment as well as instructions for use and proper disposal.

A variety of protocols including ELISA, RIA, and FACS for measuring TCCV-1 or TCCV-2 are known in the art and provide a basis for diagnosing altered or abnormal levels of TCCV-1 or TCCV-2 expression. Normal or standard values for TCCV-1 or TCCV-2 expression are established by combining body fluids or cell extracts taken from normal mammalian subjects, preferably human, with antibody to TCCV-1 or TCCV-2 under conditions suitable for complex formation. The amount of standard complex formation may be quantified by various methods, but preferably by

photometric, means. Quantities of TCCV-1 or TCCV-2 expressed in control and disease samples from biopsied tissues are compared with the standard values. Deviation between standard and subject values establishes the parameters for diagnosing disease.

In another embodiment of the invention, the polynucleotides encoding TCCV-1 or TCCV-2 may be used for diagnostic purposes. The polynucleotides which may be used include oligonucleotide sequences, complementary RNA and DNA molecules, and PNAs. The polynucleotides may be used to detect and quantitate gene expression in biopsied tissues in which expression of TCCV-1 or TCCV-2 may be correlated with disease. The diagnostic assay may be used to distinguish between absence, presence, and excess expression of TCCV-1 or TCCV-2, and to monitor regulation of TCCV-1 or TCCV-2 levels during therapeutic intervention.

In one aspect, hybridization with PCR probes which are capable of detecting polynucleotide sequences, including genomic sequences, encoding TCCV-1 or TCCV-2 or closely related molecules, may be used to identify nucleic acid sequences which encode TCCV-1 or TCCV-2. The specificity of the probe, whether it is made from a highly specific region, e.g., 10 unique nucleotides in the 5' regulatory region, or a less specific region, e.g., especially in the 3' coding region, and the stringency of the hybridization or amplification (maximal, high, intermediate, or low) will determine whether the probe identifies only naturally occurring sequences encoding TCCV-1 or TCCV-2, alleles, or related sequences.

Probes may also be used for the detection of related sequences, and should preferably contain at least 50% of the nucleotides from any of the TCCV-1 or TCCV-2 encoding sequences. The hybridization probes of the subject invention may be DNA or RNA and derived from the nucleotide sequence of SEQ ID NOs:1 or 3 or from genomic sequence including promoter, enhancer elements, and introns of the naturally occurring TCCV-1 or TCCV-2.

Means for producing specific hybridization probes for DNAs encoding TCCV-1 or TCCV-2 include the cloning of nucleic acid sequences encoding TCCV-1 or TCCV-2 or TCCV-1 or TCCV-2 derivatives into vectors for the production of mRNA probes. Such vectors are known in the art, commercially available, and may be used to synthesize RNA probes in vitro by means of the addition of the appropriate RNA polymerases and the appropriate labeled nucleotides. Hybridization probes may be labeled by a variety of reporter groups, for example, radionuclides such as ^{32}P or ^{35}S, or enzymatic labels, such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems, and the like.

Polynucleotide sequences encoding TCCV-1 or TCCV-2 may be used for the diagnosis of diseases, conditions, or disorders which are associated with expression of TCCV-1 or TCCV-2 including, but not limited to, pain; peripheral pain; peripheral neuropathies; pain caused by trauma or toxic compounds; diabetic neuropathy; cancer pain, and the like.

In order to provide a basis for the diagnosis of disease associated with expression of TCCV-1 or TCCV-2, a normal or standard profile for expression is established. This may be accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with a polynucleotide sequence, or a fragment thereof, which encodes TCCV-1 or TCCV-2, under conditions suitable for hybridization or amplification. Standard hybridization may be quantified by comparing the values obtained from normal subjects with those from an experiment where a known amount of a substantially purified polynucleotide is used. Standard values obtained from normal samples may be compared with values obtained from samples from subjects who are symptomatic for disease. Deviation between standard and subject values is used to establish the presence of disease.

Once a disease is established and a treatment protocol is initiated, hybridization assays may be repeated on a regular basis to evaluate whether the level of expression in the subject begins to approximate that which is observed in the normal subject. The results obtained from successive assays may be used to show the efficacy of treatment over a period ranging from several hours to several days to several months.

Additional diagnostic uses for oligonucleotides designed from the sequences encoding TCCV-1 or TCCV-2 may involve the use of PCR. Such oligomers may be chemically synthesized, generated enzymatically, or produced in vitro. Oligomers will preferably consist of two nucleotide sequences, one with sense orientation (5' to 3') and another with antisense (3' to 5'), employed under optimized conditions for identification of a specific gene or condition. The same two oligomers, nested sets of oligomers, or even a degenerate pool of oligomers may be employed under less stringent conditions for detection and/or quantitation of closely related DNA or RNA sequences.

Methods which may also be used to quantitate the expression of TCCV-1 or TCCV-2 include radiolabeling or biotinylating nucleotides, coamplification of a control nucleic acid, and standard curves onto which the experimental results are interpolated. (See, e.g., Melby, P. C. et al. (1993) J. Immunol. Methods, 159:235–244; and Duplaa, C. et al. (1993) Anal. Biochem. 212:229–236.) The speed of quantitation of multiple samples may be accelerated by running the assay in an ELISA format where the oligomer of interest is presented in various dilutions and a spectrophotometric or colorimetric response gives rapid quantitation.

In another embodiment of the invention, the nucleic acid sequences which encode TCCV-1 or TCCV-2 can be used to generate hybridization probes which are useful for mapping the naturally occurring genomic sequence. Fragments of TCCV-1 and TCCV-2 have been used to map these genes to the appropriate mouse and human chromosomes. The sequences may be mapped to a particular chromosome, to a specific region of a chromosome or to artificial chromosome constructions, such as human artificial chromosomes (HACs), yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), bacterial P1 constructions, or single chromosome cDNA libraries. (See, e.g., Price, C. M. (1993) Blood Rev. 7:127–134, and Trask, B. J. (1991) Trends Genet. 7:149–154.)

Fluorescent in situ hybridization (FISH) may be correlated with other physical chromosome mapping techniques and genetic map data. (See, e.g., Verma et al. (1988) Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York, N.Y.) Examples of genetic map data can be found in various scientific journals or at Online Mendelian Inheritance in Man (OMIM). Correlation between the location of the gene encoding TCCV-1 or TCCV-2 on a physical chromosomal map and a specific disease, or predisposition to a specific disease, may help delimit the region of DNA associated with that genetic disease. The nucleotide sequences of the subject invention may be used to detect differences in gene sequences between normal, carrier, or affected individuals.

In situ hybridization of chromosomal preparations and physical mapping techniques such as linkage analysis using

established chromosomal markers may be used for extending genetic maps. Often the placement of a gene on the chromosome of another mammalian species, such as mouse, may reveal associated markers even if the number or arm of a particular human chromosome is not known. New sequences can be assigned to chromosomal arms, or parts thereof, by physical mapping. This provides valuable information to investigators searching for disease genes using positional cloning or other gene discovery techniques. Once the disease or syndrome has been crudely localized by genetic linkage to a particular genomic region (see, e.g., Gatti, R. A. et al. (1988) Nature 336:577–580), any sequences mapping to that area may represent associated or regulatory genes for further investigation. The nucleotide sequence of the subject invention may also be used to detect differences in the chromosomal location due to translocation, inversion, etc. among normal, carrier, or affected individuals.

All patents, patent applications, and publications mentioned herein, whether supra or infra, are each incorporated by reference in its entirety. The broad scope of this invention is best understood with reference to the following examples, which are not intended to limit the invention to the specific embodiments described below.

EXAMPLES

Some of the standard methods are described or referenced, e.g., in Maniatis et al. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor Press; Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, (2d ed.), vols. 1–3, CSH Press, N.Y.; or Ausubel et al. (1987 and Supplements) Current Protocols in Molecular Biology, Greene/Wiley, New York; Innis et al. (eds.)(1 990) PCR Protocols: A Guide to Methods and Applications Academic Press, N.Y. Methods for protein purification include such methods as ammonium sulfate precipitation, column chromatography, electrophoresis, centrifugation, crystallization, and others. See, e.g., Ausubel et al. (1987 and periodic supplements); Deutscher (1990) "Guide to Protein Purification" in Methods in Enzymology, vol. 182, and other volumes in this series; and manufacturer's literature on use of protein purification products, e.g., Pharmacia, Piscataway, N.J., or Bio-Rad, Richmond, Calif. Combination with recombinant techniques allow fusion to appropriate segments, e.g., to a FLAG sequence or an equivalent which can be fused via a protease-removable sequence. See, e.g., Hochuli (1989) Chemische Industrie 12:69–70; Hochuli (1990) "Purification of Recombinant Proteins with Metal Chelate Absorbent" in Setlow (ed.) Genetic Engineering Principle and Methods 12:87–98, Plenum Press, N.Y.; and Crowe et al. (1992) OIAexpress: The High Level Expression & Protein Purification System QUIAGEN, Inc., Chatsworth, Calif.

Example I

Homology Search of GenBank

Searching of GenBank databases with the human T-type calcium channel subunit $\alpha 1_H$ sequence (GenBank Accession No. AF051946; and Cribbs et al. (1998) Circ. Res. 83:103–109) revealed two genomic clones from human chromosome 22 with extensive homology to $\alpha 1_H$. (GenBank Accesion Nos. AL022319 and AL008716.) BLAST results showed that these clones represented the same sequence, potentially a novel T-type Calcium channel as the $\alpha 1_G$ subunit was shown to be localized to human chromosome 17 and $\alpha 1_H$ to human chromosome 16. Additionally a further search of GenBank with the rat $\alpha 1_G$ sequence also revealed less extensive homology to the two clones above, as well as a human chromosome 17 genomic clone (GenBank Accession No. AC004590), which appeared to contain the entire human $\alpha 1_G$ sequence within 34 exons.

Comparison of the deduced exon structure of $\alpha 1_G$ with the alignments from the $\alpha 1_H$ BLAST against GenBank Accession Nos. AL022319 and AL008716 allowed the identification of many potential exons, from approximately the beginning of domain I to the end of domain IV. Due to insufficient homology with rα1G or hα1H, several exons could not initially be identified, in particular, exons corresponding to the interdomain regions. Similarly, the amino- and carboxyterminal exons could not be initially identified.

Example II

PCR Cloning and Assembly of TCCVs

PCR primers based on GenBank Accession No. AL022319 sequence were designed to clone the region from domain I to domain IV:

Sense 5' GGGCGCCATCAACTTTGACAACATC 3' (SEQ ID NO:6); and

Antisense 5' CTCACGAAGTACAGCGGCGACAC 3' (SEQ ID NO:7)

Optimized reaction conditions to produce the expected 4 kb product, per 50 μl reaction, were: each primer at 0.2μM, 1×ADVANTAGE-GC cDNA reaction buffer (Clontech, Palo Alto, Calif.), 0.2mM dNTPs, 1 μl ADVANTAGE-GC cDNA polymerase (Clontech), 1×GC MELT (Clontech), and 5 μl MARATHON-READY human brain cDNA (Clontech). Temperature and time parameters were 94°, 1 min; 95°, 10 sec, 68°, 6 min, 42 cycles; 68°, 10 min.

A band at 4 kb was excised from low-melt agarose gel, melted at 65° C. for 5 min, and subsequently ligated into the pCR2.1 TOPO vector (Invitrogen, Mountain View, Calif.) following kit instructions. The ligated vector was transformed into *E. coli* DH5α competent cells (Life Technologies, Bethesda, Md.) according to the manufacturers protocol. Two of the resulting clones, KC-1 and KC-4 were fully sequenced.

BLAST comparison of these sequences with the novel human genomic sequences AL022319 and AL008716 revealed the true exon structure for this region of the gene. This sequence was not identical to that predicted by homology with the other channels. Additionally, KC-1 had 4 mutations and KC-4 had 8 mutations relative to the genomic sequences. Of note, none of the apparent mutations in the KC-1 sequence occurred between the unique AvrII and HindIII sites. Compensation for these mutations revealed a continuous reading frame, whose deduced amino acid sequence was homologous to the $r\alpha 1_G$ and $h\alpha 1_H$ amino acid sequences corresponding to exons 6 to 31 of the $r\alpha 1_G$ sequence. Additional homology comparisons revealed that genomic clone AL008716 only contained exons 2 to 25 and genomic clone AL022319 contained, at least, exons 5 to 31.

The sequence for exons 2–7 (using the $r\alpha 1_G$ numbering of exons) was assembled electronically from the genomic sequences and used to BLAST the GenBank databases. A new chromosome 22 genomic clone was found (GenBank Accession No. AL022312), which contained exon 2 near to its 3' end. In cloning exon 1, a comparison of ral $r\alpha 1_G$ and $h\alpha 1_H$ exon 1 amino acid sequences was made, revealing a short region of amino acid homology at the 3' end. The sequence of the last 30 amino acids from $r\alpha 1_G$ exon 1 was used as query in a TFASTA search of the GenBank data-

bases. This search found a match in the new chromosome 22 genomic clone, AL022312, approximately 27 kb in the 5' direction from exon 2. This potential exon 1 had a reading frame containing the matching homology, as well as additional homology, extending to a potential initiating methionine residue. The large potential intron between exons 1 and 2 had atypical splice sites, AT . . . AC, instead of the usual GT . . . AG. The first intron of $r\alpha 1_G$ also has a similar atypical splice site. Electronic splicing of exon 1 to the previously identified exons resulted in a sequence with a continuous open reading frame.

PCR primers, described below, were designed to amplify the region from about 190 bp 5' of the likely start codon to about 120 bp 3' of the unique AvrII site:

Sense 5' CTGGGCCCTCAGCTGTTTCGTAATC 3' (SEQ ID NO:8); and

Antisense 5' GCGCTGGTCATAGCTCATCCTCCCTAGAGA 3' (SEQ ID NO:9)

Reaction conditions were the same as above, except 2.5 μl MARATHON-READY human brain cDNA (Clontech) was used as template in a 25 μl reaction, but in the absence of GC-MELT (Clontech). PCR reaction conditions were: 95° C., 1 min; 95° C., 10 sec, 68° C., 20 sec, 72° C., 4 min, 42 cycles; 72°C., 7 min. A portion of this reaction was run into a low-melt agarose gel and a band at 3 kb was excised and cloned as described above. Of four isolates sequenced, KZ-2 was found to have only one silent mutation between the 5' end and the AvrII site.

In order to identify the 3' most exons, 16 kb of genomic clone AL022319 sequence, beginning near exon 26, was run on the GENIE gene finder program, (Lawrence Berkeley National Laboratory) which predicted exons 29, 30, 31 as well as four new additional exons following exon 31. The last exon contained a stop codon in the reading frame and appeared to lack additional splice consensus sites. An additional analysis of 10 kb in the 3' direction predicted no additional exons.

PCR primers, as described below, were designed to overlap the KC-1 sequence (about 200 bp 5' of the HindIII site) and to include the coding region of the possible 3' most exon, including about 100 bp of 3' non-coding sequence:

Sense 5' GCGCTTCTTCAAGGACCGATGG 3' (SEQ ID NO:10); and Antisense 5' CCCAGGTGTGGACGAAGTATTGCT 3' (SEQ ID NO:11)

Reaction conditions for amplifying the highly GC-rich sequence were the same as above, except 2.5 μl MARATHON-READY human brain cDNA (Clontech) was used as template in a 25 μl reaction, including 1×GC-MELT (Clontech). PCR reaction conditions were: 95° C., 1 min; 95° C., 10 sec, 62° C., 20 sec, 72° C., 4 min, 42 cycles; 72° C., 5 min. A portion of this reaction was run into a low-melt agarose gel and a band at 2.1 kb was excised and cloned as above. Sequence analysis of several of these clones revealed the correct exon structure for this region, which was not entirely as predicted, and the presence of alternative 3' splice site usage in some clones, resulting in a 39 bp difference in exon 32. All clones had one or more base-substitution mutations. However, KS-6, containing the short form of exon 32, had only one silent mutation in the 5' half of the gene bounded by the unique HindIII and BamHI sites. KS-18, containing the longer form of exon 32, also had no mutations between HindIII and BamHI, whereas KS-13 was mutation free only from the BamHI site to the 3' end. Thus, two versions of the 3' end region of the gene from the HindIII site to the stop codon, differing only in the exon 32 splice variation, could be assembled from these three clones. All three clones were digested with HindIII and BamHI and the reaction products run on a low-melt agarose gel. The desired bands were excised from the gel, melted briefly at 65° C., ligated together and transformed into *E. coli* DH5α competent cells as above. The 0.9 kb fragments from KS-6 and KS-18 were separately ligated to the 5 kb fragment from KS-13 to give isolates LD-1 and LE-1 respectively.

To assemble full-length coding sequences for the two human α1I splice variants, the 3 kb KZ-2 EcoRI-AvrII fragment, the 2 kb KC-1 AvrII-HindIII fragment, either the 2 kb LD-1 or LE-1 HindIII-NotI fragment, and the 5.5 kb pCIneo (Promega) mammalian cell expression vector EcoRI-NotI fragment were prepared and ligated together as above. Of the products of these clonings, isolate LF-1 (TCCV-1) contains the full-length short exon 32 form and isolate LG-1 (TCCV-2) contains the full length long exon 32 form of the human $\alpha 1_I$ subunit.

Example III

Analysis of Splicing Patterns

Patterns of splicing at the 3' end of human and rat $\alpha 1_I$ subunit genes were investigated by PCR. Primers were designed to amplify the entire region as well as to amplify specific splice products. Primer locations were chosen, in part, to minimize differences in the rat and human sequences, so that a single primer set could be used to amplify from both templates. Primers 6066 and 6831 were designed to amplify the region from exon 31 to 35 containing the rat and human splice variations. (See FIGS. 2A–2C.)

Four forward primers (Primer Numbers 6352, 6344/88, 6495, and 6495/37) were designed from the human DNA sequences to examine specifically splicing at exon 32 and at exon 33 and to be used with reverse primer 6831. As shown in FIGS. 2A–2C, these primers were designed to span the splice sites, so that only one specific product could be amplified for each primer. The human intron sequence was considered in designing these primers to reduce the possibility of amplifying unspliced sequences.

Optimum PCR conditions were established, using plasmid templates containing the long and short forms of exon 32 and the rat and human forms of exon 33, for which the specific PCR product for each primer set was obtained only from the specific template: 94° C., 30 sec; 94° C., 10 sec, 62° C., 15 sec, 68° C., 1 min, 30 cycles; 68° C., 3 min. PCR reaction conditions were as follows: 1×ADVANTAGE cDNA PCR reaction buffer (Clontech), 0.2 mM dNTPs, 1×PCRX reagent (Life Technologies), 0.2 μM each primer, 0.2 μl 50×ADVANTAGE cDNA polymerase mix (Clontech) and 0.5 ng plasmid template in a 20 μl reaction.

To examine the presence of the various splice products in MARATHON-READY human brain cDNA (Clontech), 2.5 μl template was used in 25 μl reactions as above with 0.25 μl 50×ADVANTAGE cDNA polymerase mix (Clontech). Cycling conditions were identical, except that the annealing temperature was 63° C. for 36 or 42 cycles. Similar results were obtained at 36 or 42 cycles. The long form of exon 32 (TCCV-1) was somewhat more abundant (2 to 5 fold) than the short form (TCCV-2). In addition, only the "human" form of exon 33 was found. A PCR product corresponding to the rat $\alpha 1_I$ subunit was not detected in the human brain cDNA (See FIG. 3).

Example IV

Transfection of TSA201 Cells

TSA201 cells were plated into wells of BIOCOAT poly-D-lysine coated 6 well dishes (Becton-Dickinson, Mountain

View, Calif.) at a density of 3×10^5 cells/well two days prior to transfection or 7.5×10^5 cells/well one day prior to transfection. The medium was either the usual culture medium but without antibiotics, or, in some cases, a special low-calcium medium.

The vectors containing TCCV-1 or TCCV-2 were transfected into TSA201 cells using the LIPOFECTAMINE 2000 (Life Technologies, Bethesda, Md.) transfection kit and accompanying protocols. For each well of transfected cells, 4 μg of TCCV-1 or TCCV-2 plasmid DNA and 0.8 μg of pHook-1 DNA were combined in a tube with 250 μl of OPTI-MEM serum free medium (Life Technologies). An equal volume of diluted LIPOFECTAMINE 2000 reagent was added to each tube of diluted DNA and the mixtures were mixed and allowed to incubate at room temperature in the dark for 20 minutes. During the incubation, the medium on the cells was changed to 2.5 ml/well of DMEM with 0.1 mM MEM non-essential amino acids (Life Technologies), without serum and without antibiotics. The DNA/LIPOFECTAMINE 2000/OPTI-MEM mixture was added dropwise to cell wells while swirling the microtiter plate. The plate was returned to 37° C., 5% CO_2 for 4 to 5 hours.

Cells were resuspended and plated immediately after stopping the transfection reaction. Medium was removed from the cell wells and replaced with 2 ml of Dulbecco's Phosphate Buffered Saline (Life Technologies) without calcium or magnesium. The dish was returned to the incubator for four minutes. Cell monolayers were rinsed from the surface of the wells by trituration with a 2 ml pipet, directing the stream at the surface of the well to dislodge the cells. The resuspended cells were plated at 1:20 dilution in either regular culture medium or low calcium medium in 35 mm dishes that had been pre-coated with poly-D-lysine.

Alternatively, following the 4–5 hour incubation described above, the medium was replaced with either regular culture medium or low calcium medium and the cells were incubated overnight at 37° C. The cells were subsequently resuspended and plated as described above.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 12

<210> SEQ ID NO 1
<211> LENGTH: 6816
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (192)..(6716)

<400> SEQUENCE: 1

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tcaggtgggg ctgtcagagc tgcatccgtc cacttattgg tggagaggca ggttggggag     120

catgtaccag gcctgtcccc accacgtgcc accctctctg tcttccccag ggctcccagc     180

tcagtgtgga c atg gct gag agc gcc tcc ccg ccc tcc tca tct gca gca       230
             Met Ala Glu Ser Ala Ser Pro Pro Ser Ser Ser Ala Ala
               1               5                  10

gcc cca gcc gct gag cca gga gtc acc acg gag cag ccc gga ccc cgg       278
Ala Pro Ala Ala Glu Pro Gly Val Thr Thr Glu Gln Pro Gly Pro Arg
     15                  20                  25

agc ccc cca tcc tcc ccg cca ggc ctg gag gag cct ctg gat gga gct       326
Ser Pro Pro Ser Ser Pro Pro Gly Leu Glu Glu Pro Leu Asp Gly Ala
 30                  35                  40                  45

gat cct cat gtc cca cac cca gac ctg gcg cct att gcc ttc ttc tgc       374
Asp Pro His Val Pro His Pro Asp Leu Ala Pro Ile Ala Phe Phe Cys
                 50                  55                  60

ctg cga cag acc acc agc ccc cgg aac tgg tgc atc aag atg gtg tgc       422
Leu Arg Gln Thr Thr Ser Pro Arg Asn Trp Cys Ile Lys Met Val Cys
             65                  70                  75

aac ccg tgg ttt gaa tgt gtc agc atg ctg gtg atc ctg ctg aac tgc       470
Asn Pro Trp Phe Glu Cys Val Ser Met Leu Val Ile Leu Leu Asn Cys
         80                  85                  90

gtg aca ctt ggc atg tac cag ccg tgc gac gac atg gac tgc ctg tcc       518
Val Thr Leu Gly Met Tyr Gln Pro Cys Asp Asp Met Asp Cys Leu Ser
     95                  100                 105

gac cgc tgc aag atc ctg cag gtc ttt gat gac ttc atc ttt atc ttc       566
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Asp Arg Cys Lys Ile Leu Gln Val Phe Asp Asp Phe Ile Phe Ile Phe
110                 115                 120                 125

ttt gcc atg gag atg gtg ctc aag atg gtg gcc ctg ggg att ttt ggc      614
Phe Ala Met Glu Met Val Leu Lys Met Val Ala Leu Gly Ile Phe Gly
                130                 135                 140

aag aag tgc tac ctc ggg gac aca tgg aac cgc ctg gat ttc ttc atc      662
Lys Lys Cys Tyr Leu Gly Asp Thr Trp Asn Arg Leu Asp Phe Phe Ile
            145                 150                 155

gtc atg gca ggg atg gtc gag tac tcc ctg gac ctt cag aac atc aac      710
Val Met Ala Gly Met Val Glu Tyr Ser Leu Asp Leu Gln Asn Ile Asn
        160                 165                 170

ctg tca gcc atc cgc acc gtg cgc gtc ctg agg ccc ctc aaa gcc atc      758
Leu Ser Ala Ile Arg Thr Val Arg Val Leu Arg Pro Leu Lys Ala Ile
    175                 180                 185

aac cgc gtg ccc agt atg cgg atc ctg gtg aac ctg ctc ctg gac aca      806
Asn Arg Val Pro Ser Met Arg Ile Leu Val Asn Leu Leu Leu Asp Thr
190                 195                 200                 205

ctg ccc atg ctg ggg aat gtc ctg ctg ctc tgc ttc ttt gtc ttc ttc      854
Leu Pro Met Leu Gly Asn Val Leu Leu Leu Cys Phe Phe Val Phe Phe
                210                 215                 220

atc ttt ggc atc ata ggt gtg cag ctc tgg gcg ggc ctg ctg cgt aac      902
Ile Phe Gly Ile Ile Gly Val Gln Leu Trp Ala Gly Leu Leu Arg Asn
            225                 230                 235

cgc tgc ttc ctg gag gag aac ttc acc ata caa ggg gat gtg gcc ttg      950
Arg Cys Phe Leu Glu Glu Asn Phe Thr Ile Gln Gly Asp Val Ala Leu
        240                 245                 250

ccc cca tac tac cag ccg gag gag gat gat gag atg ccc ttc atc tgc      998
Pro Pro Tyr Tyr Gln Pro Glu Glu Asp Asp Glu Met Pro Phe Ile Cys
    255                 260                 265

tcc ctg tcg ggc gac aat ggg ata atg ggc tgc cat gag atc ccc ccg     1046
Ser Leu Ser Gly Asp Asn Gly Ile Met Gly Cys His Glu Ile Pro Pro
270                 275                 280                 285

ctc aag gag cag ggc cgt gag tgc tgc ctg tcc aag gac gac gtc tac     1094
Leu Lys Glu Gln Gly Arg Glu Cys Cys Leu Ser Lys Asp Asp Val Tyr
                290                 295                 300

gac ttt ggg gcg ggg cgc cag gac ctc aat gcc agc ggc ctc tgt gtc     1142
Asp Phe Gly Ala Gly Arg Gln Asp Leu Asn Ala Ser Gly Leu Cys Val
            305                 310                 315

aac tgg aac cgt tac tac aat gtg tgc cgc acg ggc agc gcc aac ccc     1190
Asn Trp Asn Arg Tyr Tyr Asn Val Cys Arg Thr Gly Ser Ala Asn Pro
        320                 325                 330

cac aag ggt gcc atc aac ttt gac aac atc ggt tat gct tgg att gtc     1238
His Lys Gly Ala Ile Asn Phe Asp Asn Ile Gly Tyr Ala Trp Ile Val
    335                 340                 345

atc ttc cag gtg atc act ctg gaa ggc tgg gtg gag atc atg tac tac     1286
Ile Phe Gln Val Ile Thr Leu Glu Gly Trp Val Glu Ile Met Tyr Tyr
350                 355                 360                 365

gtg atg gat gct cac tcc ttc tac aac ttc atc tac ttc atc ctg ctt     1334
Val Met Asp Ala His Ser Phe Tyr Asn Phe Ile Tyr Phe Ile Leu Leu
                370                 375                 380

atc ata gtg ggc tcc ttc ttc atg atc aac ctg tgc ctc gtt gtc ata     1382
Ile Ile Val Gly Ser Phe Phe Met Ile Asn Leu Cys Leu Val Val Ile
            385                 390                 395

gcg acc cag ttc tcg gag acc aag caa cgg gag cac cgg ctg atg ctg     1430
Ala Thr Gln Phe Ser Glu Thr Lys Gln Arg Glu His Arg Leu Met Leu
        400                 405                 410

gag cag cgg cag cgc tac ctg tcc tcc agc acg gtg gcc agc tac gcc     1478
Glu Gln Arg Gln Arg Tyr Leu Ser Ser Ser Thr Val Ala Ser Tyr Ala
    415                 420                 425
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Glu Pro Gly Asp Cys Tyr Glu Glu Ile Phe Gln Tyr Val Cys His Ile
430                 435                 440                 445

ctg cgc aag gcc aag cgc cgc gcc ctg ggc ctc tac cag gcc ctg cag     1574
Leu Arg Lys Ala Lys Arg Arg Ala Leu Gly Leu Tyr Gln Ala Leu Gln
                450                 455                 460

agc cgg cgc cag gcc ctg ggc ccg gag gcc ccg gcc ccc gcc aaa cct     1622
Ser Arg Arg Gln Ala Leu Gly Pro Glu Ala Pro Ala Pro Ala Lys Pro
            465                 470                 475

ggg ccc cac gcc aag gag ccc cgg cac tac cag ctg tgc ccg caa cat     1670
Gly Pro His Ala Lys Glu Pro Arg His Tyr Gln Leu Cys Pro Gln His
        480                 485                 490

agc ccc ctg gat gcg acg ccc cac acc ctg gtg cag ccc atc ccc gcc     1718
Ser Pro Leu Asp Ala Thr Pro His Thr Leu Val Gln Pro Ile Pro Ala
    495                 500                 505

acg ctg gct tcc gat ccc gcc agc tgc cct tgc tgc cag cat gag gac     1766
Thr Leu Ala Ser Asp Pro Ala Ser Cys Pro Cys Cys Gln His Glu Asp
510                 515                 520                 525

ggc cgg cgg ccc tcg ggc ctg ggc agc acc gac tcg ggc cag gag ggc     1814
Gly Arg Arg Pro Ser Gly Leu Gly Ser Thr Asp Ser Gly Gln Glu Gly
                530                 535                 540

tcg ggc tcc ggg agc tcc gct ggt ggc gag gac gag gcg gat ggg gac     1862
Ser Gly Ser Gly Ser Ser Ala Gly Gly Glu Asp Glu Ala Asp Gly Asp
            545                 550                 555

ggg gcc cgg agc agc gag gac gga gcc tcc tca gaa ctg ggg aag gag     1910
Gly Ala Arg Ser Ser Glu Asp Gly Ala Ser Ser Glu Leu Gly Lys Glu
        560                 565                 570

gag gag gag gag gag cag gcg gat ggg gcg gtc tgg ctg tgc ggg gat     1958
Glu Glu Glu Glu Glu Gln Ala Asp Gly Ala Val Trp Leu Cys Gly Asp
    575                 580                 585

gtg tgg cgg gag acg cga gcc aag ctg cgc ggc atc gtg gac agc aag     2006
Val Trp Arg Glu Thr Arg Ala Lys Leu Arg Gly Ile Val Asp Ser Lys
590                 595                 600                 605

tac ttc aac cgg ggc atc atg atg gcc atc ctg gtc aac acc gtc agc     2054
Tyr Phe Asn Arg Gly Ile Met Met Ala Ile Leu Val Asn Thr Val Ser
                610                 615                 620

atg ggc atc gag cac cac gag cag ccg gag gag ctg acc aac atc ctg     2102
Met Gly Ile Glu His His Glu Gln Pro Glu Glu Leu Thr Asn Ile Leu
            625                 630                 635

gag atc tgc aat gtg gtc ttc acc agc atg ttt gcc ctg gag atg atc     2150
Glu Ile Cys Asn Val Val Phe Thr Ser Met Phe Ala Leu Glu Met Ile
        640                 645                 650

ctg aag ctg gct gca ttt ggg ctc ttc gac tac ctg cgt aac ccc tac     2198
Leu Lys Leu Ala Ala Phe Gly Leu Phe Asp Tyr Leu Arg Asn Pro Tyr
    655                 660                 665

aac atc ttc gac agc atc att gtc atc atc agc atc tgg gag atc gtg     2246
Asn Ile Phe Asp Ser Ile Ile Val Ile Ile Ser Ile Trp Glu Ile Val
670                 675                 680                 685

ggg cag gcg gac ggt ggg ctg tcg gtg ctg cgg acc ttc cgg ctg ctg     2294
Gly Gln Ala Asp Gly Gly Leu Ser Val Leu Arg Thr Phe Arg Leu Leu
                690                 695                 700

cgc gtg ctg aaa ctg gtg cgc ttc atg cct gcc ctg cgg cgc cag ctc     2342
Arg Val Leu Lys Leu Val Arg Phe Met Pro Ala Leu Arg Arg Gln Leu
            705                 710                 715

gtg gtg ctc atg aag acc atg gac aac gtg gcc acc ttc tgc atg ctg     2390
Val Val Leu Met Lys Thr Met Asp Asn Val Ala Thr Phe Cys Met Leu
        720                 725                 730

ctc atg ctc ttc atc ttc atc ttc agc atc ctt ggg atg cat att ttt     2438
Leu Met Leu Phe Ile Phe Ile Phe Ser Ile Leu Gly Met His Ile Phe
    735                 740                 745
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ggc tgc aag ttc agc ctc cgc acg gac act gga gac acg gtg ccc gac     2486
Gly Cys Lys Phe Ser Leu Arg Thr Asp Thr Gly Asp Thr Val Pro Asp
750                 755                 760                 765

agg aag aac ttc gac tcc ctg ctg tgg gcc atc gtc act gtg ttc cag     2534
Arg Lys Asn Phe Asp Ser Leu Leu Trp Ala Ile Val Thr Val Phe Gln
                770                 775                 780

atc ctc acc cag gag gac tgg aac gtc gtt ctc tac aat ggc atg gcc     2582
Ile Leu Thr Gln Glu Asp Trp Asn Val Val Leu Tyr Asn Gly Met Ala
            785                 790                 795

tcc act tct ccc tgg gcc tcc ctc tac ttt gtc gcc ctc atg acc ttc     2630
Ser Thr Ser Pro Trp Ala Ser Leu Tyr Phe Val Ala Leu Met Thr Phe
        800                 805                 810

ggc aac tat gtg ctc ttc aac ctg ctg gtg gcc atc ctg gtg gag ggc     2678
Gly Asn Tyr Val Leu Phe Asn Leu Leu Val Ala Ile Leu Val Glu Gly
    815                 820                 825

ttc cag gcg gag ggt gac gcc aat cgc tcc tac tcg gac gag gac cag     2726
Phe Gln Ala Glu Gly Asp Ala Asn Arg Ser Tyr Ser Asp Glu Asp Gln
830                 835                 840                 845

agc tca tcc aac ata gaa gag ttt gat aag ctc cag gaa ggc ctg gac     2774
Ser Ser Ser Asn Ile Glu Glu Phe Asp Lys Leu Gln Glu Gly Leu Asp
                850                 855                 860

agc agc gga gat ccc aag ctc tgc cca atc ccc atg acc ccc aat ggg     2822
Ser Ser Gly Asp Pro Lys Leu Cys Pro Ile Pro Met Thr Pro Asn Gly
            865                 870                 875

cac ctg gac ccc agt ctc cca ctg ggt ggg cac cta ggt cct gct ggg     2870
His Leu Asp Pro Ser Leu Pro Leu Gly Gly His Leu Gly Pro Ala Gly
        880                 885                 890

gct gcg gga cct gcc ccc cga ctc tca ctg cag ccg gac ccc atg ctg     2918
Ala Ala Gly Pro Ala Pro Arg Leu Ser Leu Gln Pro Asp Pro Met Leu
    895                 900                 905

gtg gcc ctg ggc tcc cga aag agc agt gtc atg tct cta ggg agg atg     2966
Val Ala Leu Gly Ser Arg Lys Ser Ser Val Met Ser Leu Gly Arg Met
910                 915                 920                 925

agc tat gac cag cgc tcc ctg tcc agc tcc cgg agc tcc tac tac ggg     3014
Ser Tyr Asp Gln Arg Ser Leu Ser Ser Ser Arg Ser Ser Tyr Tyr Gly
                930                 935                 940

cca tgg ggc cgc agc gcg gcc tgg gcc agc cgt cgc tcc agc tgg aac     3062
Pro Trp Gly Arg Ser Ala Ala Trp Ala Ser Arg Arg Ser Ser Trp Asn
            945                 950                 955

agc ctc aag cac aag ccg ccg tcg gcg gag cat gag tcc ctg ctc tct     3110
Ser Leu Lys His Lys Pro Pro Ser Ala Glu His Glu Ser Leu Leu Ser
        960                 965                 970

gcg gag cgc ggc ggc ggc gcc cgg gtc tgc gag gtt gcc gcg gac gag     3158
Ala Glu Arg Gly Gly Gly Ala Arg Val Cys Glu Val Ala Ala Asp Glu
    975                 980                 985

ggg ccg ccg cgg gcc gca ccc ctg cac acc cca cac gcc cac cac att     3206
Gly Pro Pro Arg Ala Ala Pro Leu His Thr Pro His Ala His His Ile
990                 995                 1000                1005

cat cac ggg ccc cat ctg gcg cac cgc cac cgc cac cac cgc cgg acg     3254
His His Gly Pro His Leu Ala His Arg His Arg His His Arg Arg Thr
                1010                1015                1020

ctg tcc ctc gac aac agg gac tcg gtg gac ctg gcc gag ctg gtg ccc     3302
Leu Ser Leu Asp Asn Arg Asp Ser Val Asp Leu Ala Glu Leu Val Pro
            1025                1030                1035

gcg gtg ggc gcc cac ccc cgg gcc gcc tgg agg gcg gca ggc ccg gcc     3350
Ala Val Gly Ala His Pro Arg Ala Ala Trp Arg Ala Ala Gly Pro Ala
        1040                1045                1050

ccc ggg cat gag gac tgc aat ggc agg atg ccc agc atc gcc aaa gac     3398
Pro Gly His Glu Asp Cys Asn Gly Arg Met Pro Ser Ile Ala Lys Asp
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    1055                1060                1065

gtc ttc acc aag atg ggc gac cgc ggg gat cgc ggg gag gat gag gag     3446
Val Phe Thr Lys Met Gly Asp Arg Gly Asp Arg Gly Glu Asp Glu Glu
1070                1075                1080                1085

gaa atc gac tac acc ctg tgc ttc cgc gtc cgc aag atg atc gac gtc     3494
Glu Ile Asp Tyr Thr Leu Cys Phe Arg Val Arg Lys Met Ile Asp Val
                1090                1095                1100

tat aag ccc gac tgg tgc gag gtc cgc gaa gac tgg tct gtc tac ctc     3542
Tyr Lys Pro Asp Trp Cys Glu Val Arg Glu Asp Trp Ser Val Tyr Leu
            1105                1110                1115

ttc tct ccc gag aac agg ttc cgg gtc ctg tgt cag acc att att gcc     3590
Phe Ser Pro Glu Asn Arg Phe Arg Val Leu Cys Gln Thr Ile Ile Ala
        1120                1125                1130

cac aaa ctc ttc gac tac gtc gtc ctg gcc ttc atc ttt ctc aac tgc     3638
His Lys Leu Phe Asp Tyr Val Val Leu Ala Phe Ile Phe Leu Asn Cys
    1135                1140                1145

atc acc atc gcc ctg gag cgg cct cag atc gag gcc ggc agc acc gaa     3686
Ile Thr Ile Ala Leu Glu Arg Pro Gln Ile Glu Ala Gly Ser Thr Glu
1150                1155                1160                1165

cgc atc ttt ctc acc gtg tcc aac tac atc ttc acg gcc atc ttc gtg     3734
Arg Ile Phe Leu Thr Val Ser Asn Tyr Ile Phe Thr Ala Ile Phe Val
                1170                1175                1180

ggc gag atg aca ttg aag gta gtc tcg ctg ggc ctg tac ttc ggc gag     3782
Gly Glu Met Thr Leu Lys Val Val Ser Leu Gly Leu Tyr Phe Gly Glu
            1185                1190                1195

cag gcg tac cta cgc agc agc tgg aac gtg ctg gat ggc ttt ctt gtc     3830
Gln Ala Tyr Leu Arg Ser Ser Trp Asn Val Leu Asp Gly Phe Leu Val
        1200                1205                1210

ttc gtg tcc atc atc gac atc gtg gtg tcc ctg gcc tca gcc ggg gga     3878
Phe Val Ser Ile Ile Asp Ile Val Val Ser Leu Ala Ser Ala Gly Gly
    1215                1220                1225

gcc aag atc ttg ggg gtc ctc cga gtc ttg cgg ctc ctg cgc acc cta     3926
Ala Lys Ile Leu Gly Val Leu Arg Val Leu Arg Leu Leu Arg Thr Leu
1230                1235                1240                1245

cgc ccc ctg cgt gtc atc agc cgg gcg ccg ggc ctg aag ctg gtg gtg     3974
Arg Pro Leu Arg Val Ile Ser Arg Ala Pro Gly Leu Lys Leu Val Val
                1250                1255                1260

gag aca ctc atc tcc tcc ctc aag ccc atc ggc aac atc gtg ctc atc     4022
Glu Thr Leu Ile Ser Ser Leu Lys Pro Ile Gly Asn Ile Val Leu Ile
            1265                1270                1275

tgc tgt gcc ttc ttc atc atc ttt ggc atc ctg gga gtg cag ctc ttc     4070
Cys Cys Ala Phe Phe Ile Ile Phe Gly Ile Leu Gly Val Gln Leu Phe
        1280                1285                1290

aag ggc aag ttc tac cac tgt ctg ggc gtg gac acc cgc aac atc acc     4118
Lys Gly Lys Phe Tyr His Cys Leu Gly Val Asp Thr Arg Asn Ile Thr
    1295                1300                1305

aac cgc tcg gac tgc atg gcc gcc aac tac cgc tgg gtc cat cac aaa     4166
Asn Arg Ser Asp Cys Met Ala Ala Asn Tyr Arg Trp Val His His Lys
1310                1315                1320                1325

tac aac ttc gac aac ctg ggc cag gct ctg atg tcc ctc ttt gtc ctg     4214
Tyr Asn Phe Asp Asn Leu Gly Gln Ala Leu Met Ser Leu Phe Val Leu
                1330                1335                1340

gca tcc aag gat ggt tgg gtg aac atc atg tac aat gga ctg gat gct     4262
Ala Ser Lys Asp Gly Trp Val Asn Ile Met Tyr Asn Gly Leu Asp Ala
            1345                1350                1355

gtt gct gtg gac cag cag cct gtg acc aac cac aac ccc tgg atg ctg     4310
Val Ala Val Asp Gln Gln Pro Val Thr Asn His Asn Pro Trp Met Leu
        1360                1365                1370

ctg tac ttc atc tcc ttc ctg ctc atc gtc agc ttc ttt gtg ctc aac     4358
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Leu Tyr Phe Ile Ser Phe Leu Leu Ile Val Ser Phe Phe Val Leu Asn
    1375                1380                1385

atg ttt gtg ggt gtc gtg gtg gag aac ttc cac aag tgc cgg cag cac     4406
Met Phe Val Gly Val Val Val Glu Asn Phe His Lys Cys Arg Gln His
1390                1395                1400                1405

cag gag gct gaa gag gca cgg cgg cgt gag gag aag cgg ctg cgg cgc     4454
Gln Glu Ala Glu Glu Ala Arg Arg Arg Glu Glu Lys Arg Leu Arg Arg
                1410                1415                1420

ctg gag aag aag cgc cgg aag gcc cag cgg ctg ccc tac tat gcc acc     4502
Leu Glu Lys Lys Arg Arg Lys Ala Gln Arg Leu Pro Tyr Tyr Ala Thr
            1425                1430                1435

tat tgt cac acc cgg ctg ctc atc cac tcc atg tgc acc agc cac tac     4550
Tyr Cys His Thr Arg Leu Leu Ile His Ser Met Cys Thr Ser His Tyr
        1440                1445                1450

ctg gac atc ttc atc acc ttc atc atc tgc ctc aac gtg gtc acc atg     4598
Leu Asp Ile Phe Ile Thr Phe Ile Ile Cys Leu Asn Val Val Thr Met
    1455                1460                1465

tcc ctg gag cac tac aat cag ccc acg tcc ctg gag aca gcc ctc aag     4646
Ser Leu Glu His Tyr Asn Gln Pro Thr Ser Leu Glu Thr Ala Leu Lys
1470                1475                1480                1485

tac tgc aac tat atg ttc acc act gtc ttt gtg ctg gag gct gtg ctg     4694
Tyr Cys Asn Tyr Met Phe Thr Thr Val Phe Val Leu Glu Ala Val Leu
                1490                1495                1500

aag ctg gtg gca ttt ggt ctg agg cgc ttc ttc aag gac cga tgg aac     4742
Lys Leu Val Ala Phe Gly Leu Arg Arg Phe Phe Lys Asp Arg Trp Asn
            1505                1510                1515

cag ctg gac ctg gcc att gtg cta ctg tca gtc atg ggc atc acc ctg     4790
Gln Leu Asp Leu Ala Ile Val Leu Leu Ser Val Met Gly Ile Thr Leu
        1520                1525                1530

gag gag atc gag atc aat gcg gcc ctg ccc atc aat ccc acc atc atc     4838
Glu Glu Ile Glu Ile Asn Ala Ala Leu Pro Ile Asn Pro Thr Ile Ile
    1535                1540                1545

cgc atc atg agg gtt ctg cgc att gcc cga gtg ctg aag ctg ttg aag     4886
Arg Ile Met Arg Val Leu Arg Ile Ala Arg Val Leu Lys Leu Leu Lys
1550                1555                1560                1565

atg gcc aca gga atg cgg gcc ctg ctg gac acg gtg gtg caa gct ttg     4934
Met Ala Thr Gly Met Arg Ala Leu Leu Asp Thr Val Val Gln Ala Leu
                1570                1575                1580

ccc cag gtg ggc aac ctg ggc ctc ctc ttc atg ctg ctc ttc ttc atc     4982
Pro Gln Val Gly Asn Leu Gly Leu Leu Phe Met Leu Leu Phe Phe Ile
            1585                1590                1595

tat gct gct ctc ggg gtg gag ctc ttt ggg aag ctg gtc tgc aac gac     5030
Tyr Ala Ala Leu Gly Val Glu Leu Phe Gly Lys Leu Val Cys Asn Asp
        1600                1605                1610

gag aac ccg tgc gag ggc atg agc cgg cat gcc acc ttc gag aac ttc     5078
Glu Asn Pro Cys Glu Gly Met Ser Arg His Ala Thr Phe Glu Asn Phe
    1615                1620                1625

ggc atg gcc ttc ctc aca ctc ttc cag gtc tcc acg ggt gac aac tgg     5126
Gly Met Ala Phe Leu Thr Leu Phe Gln Val Ser Thr Gly Asp Asn Trp
1630                1635                1640                1645

aac ggg atc atg aag gac acg ctg cgg gac tgc acc cac gac gag cgc     5174
Asn Gly Ile Met Lys Asp Thr Leu Arg Asp Cys Thr His Asp Glu Arg
                1650                1655                1660

agc tgc ctg agc agc ctg cag ttt gtg tcg ccg ctg tac ttc gtg agc     5222
Ser Cys Leu Ser Ser Leu Gln Phe Val Ser Pro Leu Tyr Phe Val Ser
            1665                1670                1675

ttc gtg ctc acc gcg cag ttc gtg ctc atc aac gtg gtg gtg gct gtg     5270
Phe Val Leu Thr Ala Gln Phe Val Leu Ile Asn Val Val Val Ala Val
        1680                1685                1690
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ctc atg aag cac ctg gac gac agc aac aag gag gcg cag gag gac gcc     5318
Leu Met Lys His Leu Asp Asp Ser Asn Lys Glu Ala Gln Glu Asp Ala
    1695                1700                1705

gag atg gat gcc gag ctc gag ctg gag atg gcc cat ggc ctg ggc cct     5366
Glu Met Asp Ala Glu Leu Glu Leu Glu Met Ala His Gly Leu Gly Pro
1710                1715                1720                1725

ggc ccg agg ctg cct acc ggc tcc ccg ggc gcc cct ggc cga ggg ccg     5414
Gly Pro Arg Leu Pro Thr Gly Ser Pro Gly Ala Pro Gly Arg Gly Pro
                1730                1735                1740

gga ggg gcg ggc ggc ggg ggc gac acc gag ggc ggc ttg tgc cgg cgc     5462
Gly Gly Ala Gly Gly Gly Gly Asp Thr Glu Gly Gly Leu Cys Arg Arg
            1745                1750                1755

tgc tac tcg cct gcc cag gac tcc ttg gag ggg gag ctg acc atc atc     5510
Cys Tyr Ser Pro Ala Gln Asp Ser Leu Glu Gly Glu Leu Thr Ile Ile
        1760                1765                1770

gac aac ctg tcg ggc tcc atc ttc cac cac tac tcc tcg cct gcc ggc     5558
Asp Asn Leu Ser Gly Ser Ile Phe His His Tyr Ser Ser Pro Ala Gly
    1775                1780                1785

tgc aag aag tgt cac cac gac aag caa gag gtg cag ctg gct gag acg     5606
Cys Lys Lys Cys His His Asp Lys Gln Glu Val Gln Leu Ala Glu Thr
1790                1795                1800                1805

gag gcc ttc tcc ctg aac tca gac agg tcc tcg tcc atc ctg ctg ggt     5654
Glu Ala Phe Ser Leu Asn Ser Asp Arg Ser Ser Ser Ile Leu Leu Gly
                1810                1815                1820

gac gac ctg agt ctc gag gac ccc aca gcc tgc cca cct ggc cgc aag     5702
Asp Asp Leu Ser Leu Glu Asp Pro Thr Ala Cys Pro Pro Gly Arg Lys
            1825                1830                1835

gac agc aag ggt gag ctg gac cca cct gag ccc atg cgt gtg gga gac     5750
Asp Ser Lys Gly Glu Leu Asp Pro Pro Glu Pro Met Arg Val Gly Asp
        1840                1845                1850

ctg ggc gaa tgc ttc ttc ccc ttg tcc tct acg gcc gtc tcg ccg gat     5798
Leu Gly Glu Cys Phe Phe Pro Leu Ser Ser Thr Ala Val Ser Pro Asp
    1855                1860                1865

cca gag aac ttc ctg tgt gag atg gag gag atc cca ttc aac cct gtc     5846
Pro Glu Asn Phe Leu Cys Glu Met Glu Glu Ile Pro Phe Asn Pro Val
1870                1875                1880                1885

cgg tcc tgg ctg aaa cat gac agc agt caa gca ccc cca agt ccc ttc     5894
Arg Ser Trp Leu Lys His Asp Ser Ser Gln Ala Pro Pro Ser Pro Phe
                1890                1895                1900

tcc ccg gat gcc tcc agc cct ctc ctg ccc atg cca gcc gag ttc ttc     5942
Ser Pro Asp Ala Ser Ser Pro Leu Leu Pro Met Pro Ala Glu Phe Phe
            1905                1910                1915

cac cct gca gtg tct gcc agc cag aaa ggc cca gaa aag ggc act ggc     5990
His Pro Ala Val Ser Ala Ser Gln Lys Gly Pro Glu Lys Gly Thr Gly
        1920                1925                1930

act gga acc ctc ccc aag att gcg ctg cag ggc tcc tgg gca tct ctg     6038
Thr Gly Thr Leu Pro Lys Ile Ala Leu Gln Gly Ser Trp Ala Ser Leu
    1935                1940                1945

cgg tca cca agg gtc aac tgt acc ctc ctc cgg cag gcc acc ggg agc     6086
Arg Ser Pro Arg Val Asn Cys Thr Leu Leu Arg Gln Ala Thr Gly Ser
1950                1955                1960                1965

gac acg tcg ctg gac gcc agc ccc agc agc tcc gcg ggc agc ctg cag     6134
Asp Thr Ser Leu Asp Ala Ser Pro Ser Ser Ser Ala Gly Ser Leu Gln
                1970                1975                1980

acc acg ctc gag gac agc ctg acc ctg agc gac agc ccc cgg cgt gcc     6182
Thr Thr Leu Glu Asp Ser Leu Thr Leu Ser Asp Ser Pro Arg Arg Ala
            1985                1990                1995

ctg ggg ccg ccc gcg cct gct cca gga ccc cgg gcc ggc ctg tcc ccc     6230
Leu Gly Pro Pro Ala Pro Ala Pro Gly Pro Arg Ala Gly Leu Ser Pro
        2000                2005                2010
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gcc gct cgc cgc cgc ctg agc ctg cgc ggc cgg ggc ctc ttc agc ctg      6278
Ala Ala Arg Arg Arg Leu Ser Leu Arg Gly Arg Gly Leu Phe Ser Leu
    2015                2020                2025

cgg ggg ctg cgg gcg cat cag cgc agc cac agc agc ggg ggc tcc acc      6326
Arg Gly Leu Arg Ala His Gln Arg Ser His Ser Ser Gly Gly Ser Thr
2030                2035                2040                2045

agc ccg ggc tgc acc cac cac gac tcc atg gac ccc tcg gac gag gag      6374
Ser Pro Gly Cys Thr His His Asp Ser Met Asp Pro Ser Asp Glu Glu
                2050                2055                2060

ggc cgc ggt ggc gcg ggc ggc ggg ggc gcg ggc agc gag cac tcg gag      6422
Gly Arg Gly Gly Ala Gly Gly Gly Gly Ala Gly Ser Glu His Ser Glu
            2065                2070                2075

acc ctc agc agc ctc tcg ctc acc tcc ctc ttc tgc ccg ccg ccc ccg      6470
Thr Leu Ser Ser Leu Ser Leu Thr Ser Leu Phe Cys Pro Pro Pro Pro
        2080                2085                2090

ccg cca gcc ccc ggc ctc acg ccc gcc agg aag ttc agc agc acc agc      6518
Pro Pro Ala Pro Gly Leu Thr Pro Ala Arg Lys Phe Ser Ser Thr Ser
    2095                2100                2105

agc ctg gcc gcc ccc ggc cgc ccc cac gcc gcc gcc ctg gcc cac ggc      6566
Ser Leu Ala Ala Pro Gly Arg Pro His Ala Ala Ala Leu Ala His Gly
2110                2115                2120                2125

ctg gcc cgg agc ccc tcg tgg gcc gcg gac cgc agc aag gac ccc ccc      6614
Leu Ala Arg Ser Pro Ser Trp Ala Ala Asp Arg Ser Lys Asp Pro Pro
                2130                2135                2140

ggc cgg gca ccg ctg ccc atg ggc ctg ggc ccc ttg gcg ccc ccg ccg      6662
Gly Arg Ala Pro Leu Pro Met Gly Leu Gly Pro Leu Ala Pro Pro Pro
            2145                2150                2155

caa ccg ctc ccc gga gag ctg gag ccg gga gac gcc gcc agc aag agg      6710
Gln Pro Leu Pro Gly Glu Leu Glu Pro Gly Asp Ala Ala Ser Lys Arg
        2160                2165                2170

aag aga tgagggtcgc aggggccccc ggccgcccac cgcccgcccc gtctcacctt      6766
Lys Arg
    2175

ctttacctca ggagccagga gcagacagca atacttcgtc cacacctggg              6816


<210> SEQ ID NO 2
<211> LENGTH: 2175
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

Met Ala Glu Ser Ala Ser Pro Pro Ser Ser Ser Ala Ala Ala Pro Ala
  1               5                  10                  15

Ala Glu Pro Gly Val Thr Thr Glu Gln Pro Gly Pro Arg Ser Pro Pro
             20                  25                  30

Ser Ser Pro Pro Gly Leu Glu Glu Pro Leu Asp Gly Ala Asp Pro His
         35                  40                  45

Val Pro His Pro Asp Leu Ala Pro Ile Ala Phe Phe Cys Leu Arg Gln
     50                  55                  60

Thr Thr Ser Pro Arg Asn Trp Cys Ile Lys Met Val Cys Asn Pro Trp
 65                  70                  75                  80

Phe Glu Cys Val Ser Met Leu Val Ile Leu Leu Asn Cys Val Thr Leu
                 85                  90                  95

Gly Met Tyr Gln Pro Cys Asp Asp Met Asp Cys Leu Ser Asp Arg Cys
            100                 105                 110

Lys Ile Leu Gln Val Phe Asp Asp Phe Ile Phe Ile Phe Phe Ala Met
        115                 120                 125
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Glu Met Val Leu Lys Met Val Ala Leu Gly Ile Phe Gly Lys Lys Cys
    130                 135                 140

Tyr Leu Gly Asp Thr Trp Asn Arg Leu Asp Phe Phe Ile Val Met Ala
145                 150                 155                 160

Gly Met Val Glu Tyr Ser Leu Asp Leu Gln Asn Ile Asn Leu Ser Ala
                165                 170                 175

Ile Arg Thr Val Arg Val Leu Arg Pro Leu Lys Ala Ile Asn Arg Val
            180                 185                 190

Pro Ser Met Arg Ile Leu Val Asn Leu Leu Leu Asp Thr Leu Pro Met
        195                 200                 205

Leu Gly Asn Val Leu Leu Leu Cys Phe Phe Val Phe Phe Ile Phe Gly
    210                 215                 220

Ile Ile Gly Val Gln Leu Trp Ala Gly Leu Leu Arg Asn Arg Cys Phe
225                 230                 235                 240

Leu Glu Glu Asn Phe Thr Ile Gln Gly Asp Val Ala Leu Pro Pro Tyr
                245                 250                 255

Tyr Gln Pro Glu Glu Asp Asp Glu Met Pro Phe Ile Cys Ser Leu Ser
            260                 265                 270

Gly Asp Asn Gly Ile Met Gly Cys His Glu Ile Pro Pro Leu Lys Glu
        275                 280                 285

Gln Gly Arg Glu Cys Cys Leu Ser Lys Asp Asp Val Tyr Asp Phe Gly
    290                 295                 300

Ala Gly Arg Gln Asp Leu Asn Ala Ser Gly Leu Cys Val Asn Trp Asn
305                 310                 315                 320

Arg Tyr Tyr Asn Val Cys Arg Thr Gly Ser Ala Asn Pro His Lys Gly
                325                 330                 335

Ala Ile Asn Phe Asp Asn Ile Gly Tyr Ala Trp Ile Val Ile Phe Gln
            340                 345                 350

Val Ile Thr Leu Glu Gly Trp Val Glu Ile Met Tyr Tyr Val Met Asp
        355                 360                 365

Ala His Ser Phe Tyr Asn Phe Ile Tyr Phe Ile Leu Leu Ile Ile Val
    370                 375                 380

Gly Ser Phe Phe Met Ile Asn Leu Cys Leu Val Val Ile Ala Thr Gln
385                 390                 395                 400

Phe Ser Glu Thr Lys Gln Arg Glu His Arg Leu Met Leu Glu Gln Arg
                405                 410                 415

Gln Arg Tyr Leu Ser Ser Ser Thr Val Ala Ser Tyr Ala Glu Pro Gly
            420                 425                 430

Asp Cys Tyr Glu Glu Ile Phe Gln Tyr Val Cys His Ile Leu Arg Lys
        435                 440                 445

Ala Lys Arg Arg Ala Leu Gly Leu Tyr Gln Ala Leu Gln Ser Arg Arg
    450                 455                 460

Gln Ala Leu Gly Pro Glu Ala Pro Ala Pro Ala Lys Pro Gly Pro His
465                 470                 475                 480

Ala Lys Glu Pro Arg His Tyr Gln Leu Cys Pro Gln His Ser Pro Leu
                485                 490                 495

Asp Ala Thr Pro His Thr Leu Val Gln Pro Ile Pro Ala Thr Leu Ala
            500                 505                 510

Ser Asp Pro Ala Ser Cys Pro Cys Cys Gln His Glu Asp Gly Arg Arg
        515                 520                 525

Pro Ser Gly Leu Gly Ser Thr Asp Ser Gly Gln Glu Gly Ser Gly Ser
    530                 535                 540

Gly Ser Ser Ala Gly Gly Glu Asp Glu Ala Asp Gly Asp Gly Ala Arg
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545                 550                 555                 560

Ser Ser Glu Asp Gly Ala Ser Ser Glu Leu Gly Lys Glu Glu Glu Glu
                565                 570                 575

Glu Glu Gln Ala Asp Gly Ala Val Trp Leu Cys Gly Asp Val Trp Arg
            580                 585                 590

Glu Thr Arg Ala Lys Leu Arg Gly Ile Val Asp Ser Lys Tyr Phe Asn
        595                 600                 605

Arg Gly Ile Met Met Ala Ile Leu Val Asn Thr Val Ser Met Gly Ile
    610                 615                 620

Glu His His Glu Gln Pro Glu Glu Leu Thr Asn Ile Leu Glu Ile Cys
625                 630                 635                 640

Asn Val Val Phe Thr Ser Met Phe Ala Leu Glu Met Ile Leu Lys Leu
                645                 650                 655

Ala Ala Phe Gly Leu Phe Asp Tyr Leu Arg Asn Pro Tyr Asn Ile Phe
            660                 665                 670

Asp Ser Ile Ile Val Ile Ile Ser Ile Trp Glu Ile Val Gly Gln Ala
        675                 680                 685

Asp Gly Gly Leu Ser Val Leu Arg Thr Phe Arg Leu Leu Arg Val Leu
    690                 695                 700

Lys Leu Val Arg Phe Met Pro Ala Leu Arg Arg Gln Leu Val Val Leu
705                 710                 715                 720

Met Lys Thr Met Asp Asn Val Ala Thr Phe Cys Met Leu Leu Met Leu
                725                 730                 735

Phe Ile Phe Ile Phe Ser Ile Leu Gly Met His Ile Phe Gly Cys Lys
            740                 745                 750

Phe Ser Leu Arg Thr Asp Thr Gly Asp Thr Val Pro Asp Arg Lys Asn
        755                 760                 765

Phe Asp Ser Leu Leu Trp Ala Ile Val Thr Val Phe Gln Ile Leu Thr
    770                 775                 780

Gln Glu Asp Trp Asn Val Val Leu Tyr Asn Gly Met Ala Ser Thr Ser
785                 790                 795                 800

Pro Trp Ala Ser Leu Tyr Phe Val Ala Leu Met Thr Phe Gly Asn Tyr
                805                 810                 815

Val Leu Phe Asn Leu Leu Val Ala Ile Leu Val Glu Gly Phe Gln Ala
            820                 825                 830

Glu Gly Asp Ala Asn Arg Ser Tyr Ser Asp Glu Asp Gln Ser Ser Ser
        835                 840                 845

Asn Ile Glu Glu Phe Asp Lys Leu Gln Glu Gly Leu Asp Ser Ser Gly
    850                 855                 860

Asp Pro Lys Leu Cys Pro Ile Pro Met Thr Pro Asn Gly His Leu Asp
865                 870                 875                 880

Pro Ser Leu Pro Leu Gly Gly His Leu Gly Pro Ala Gly Ala Ala Gly
                885                 890                 895

Pro Ala Pro Arg Leu Ser Leu Gln Pro Asp Pro Met Leu Val Ala Leu
            900                 905                 910

Gly Ser Arg Lys Ser Ser Val Met Ser Leu Gly Arg Met Ser Tyr Asp
        915                 920                 925

Gln Arg Ser Leu Ser Ser Ser Arg Ser Ser Tyr Tyr Gly Pro Trp Gly
    930                 935                 940

Arg Ser Ala Ala Trp Ala Ser Arg Arg Ser Ser Trp Asn Ser Leu Lys
945                 950                 955                 960

His Lys Pro Pro Ser Ala Glu His Glu Ser Leu Leu Ser Ala Glu Arg
                965                 970                 975
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Gly Gly Gly Ala Arg Val Cys Glu Val Ala Ala Asp Glu Gly Pro Pro
980 985 990

Arg Ala Ala Pro Leu His Thr Pro His Ala His His Ile His His Gly
995 1000 1005

Pro His Leu Ala His Arg His Arg His His Arg Arg Thr Leu Ser Leu
1010 1015 1020

Asp Asn Arg Asp Ser Val Asp Leu Ala Glu Leu Val Pro Ala Val Gly
1025 1030 1035 1040

Ala His Pro Arg Ala Ala Trp Arg Ala Ala Gly Pro Ala Pro Gly His
1045 1050 1055

Glu Asp Cys Asn Gly Arg Met Pro Ser Ile Ala Lys Asp Val Phe Thr
1060 1065 1070

Lys Met Gly Asp Arg Gly Asp Arg Gly Glu Asp Glu Glu Glu Ile Asp
1075 1080 1085

Tyr Thr Leu Cys Phe Arg Val Arg Lys Met Ile Asp Val Tyr Lys Pro
1090 1095 1100

Asp Trp Cys Glu Val Arg Glu Asp Trp Ser Val Tyr Leu Phe Ser Pro
1105 1110 1115 1120

Glu Asn Arg Phe Arg Val Leu Cys Gln Thr Ile Ile Ala His Lys Leu
1125 1130 1135

Phe Asp Tyr Val Val Leu Ala Phe Ile Phe Leu Asn Cys Ile Thr Ile
1140 1145 1150

Ala Leu Glu Arg Pro Gln Ile Glu Ala Gly Ser Thr Glu Arg Ile Phe
1155 1160 1165

Leu Thr Val Ser Asn Tyr Ile Phe Thr Ala Ile Phe Val Gly Glu Met
1170 1175 1180

Thr Leu Lys Val Val Ser Leu Gly Leu Tyr Phe Gly Glu Gln Ala Tyr
1185 1190 1195 1200

Leu Arg Ser Ser Trp Asn Val Leu Asp Gly Phe Leu Val Phe Val Ser
1205 1210 1215

Ile Ile Asp Ile Val Val Ser Leu Ala Ser Ala Gly Gly Ala Lys Ile
1220 1225 1230

Leu Gly Val Leu Arg Val Leu Arg Leu Leu Arg Thr Leu Arg Pro Leu
1235 1240 1245

Arg Val Ile Ser Arg Ala Pro Gly Leu Lys Leu Val Val Glu Thr Leu
1250 1255 1260

Ile Ser Ser Leu Lys Pro Ile Gly Asn Ile Val Leu Ile Cys Cys Ala
1265 1270 1275 1280

Phe Phe Ile Ile Phe Gly Ile Leu Gly Val Gln Leu Phe Lys Gly Lys
1285 1290 1295

Phe Tyr His Cys Leu Gly Val Asp Thr Arg Asn Ile Thr Asn Arg Ser
1300 1305 1310

Asp Cys Met Ala Ala Asn Tyr Arg Trp Val His His Lys Tyr Asn Phe
1315 1320 1325

Asp Asn Leu Gly Gln Ala Leu Met Ser Leu Phe Val Leu Ala Ser Lys
1330 1335 1340

Asp Gly Trp Val Asn Ile Met Tyr Asn Gly Leu Asp Ala Val Ala Val
1345 1350 1355 1360

Asp Gln Gln Pro Val Thr Asn His Asn Pro Trp Met Leu Leu Tyr Phe
1365 1370 1375

Ile Ser Phe Leu Leu Ile Val Ser Phe Phe Val Leu Asn Met Phe Val
1380 1385 1390

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Gly Val Val Val Glu Asn Phe His Lys Cys Arg Gln His Gln Glu Ala
1395 1400 1405

Glu Glu Ala Arg Arg Arg Glu Glu Lys Arg Leu Arg Arg Leu Glu Lys
1410 1415 1420

Lys Arg Arg Lys Ala Gln Arg Leu Pro Tyr Tyr Ala Thr Tyr Cys His
1425 1430 1435 1440

Thr Arg Leu Leu Ile His Ser Met Cys Thr Ser His Tyr Leu Asp Ile
1445 1450 1455

Phe Ile Thr Phe Ile Ile Cys Leu Asn Val Val Thr Met Ser Leu Glu
1460 1465 1470

His Tyr Asn Gln Pro Thr Ser Leu Glu Thr Ala Leu Lys Tyr Cys Asn
1475 1480 1485

Tyr Met Phe Thr Thr Val Phe Val Leu Glu Ala Val Leu Lys Leu Val
1490 1495 1500

Ala Phe Gly Leu Arg Arg Phe Phe Lys Asp Arg Trp Asn Gln Leu Asp
1505 1510 1515 1520

Leu Ala Ile Val Leu Leu Ser Val Met Gly Ile Thr Leu Glu Glu Ile
1525 1530 1535

Glu Ile Asn Ala Ala Leu Pro Ile Asn Pro Thr Ile Ile Arg Ile Met
1540 1545 1550

Arg Val Leu Arg Ile Ala Arg Val Leu Lys Leu Leu Lys Met Ala Thr
1555 1560 1565

Gly Met Arg Ala Leu Leu Asp Thr Val Val Gln Ala Leu Pro Gln Val
1570 1575 1580

Gly Asn Leu Gly Leu Leu Phe Met Leu Leu Phe Phe Ile Tyr Ala Ala
1585 1590 1595 1600

Leu Gly Val Glu Leu Phe Gly Lys Leu Val Cys Asn Asp Glu Asn Pro
1605 1610 1615

Cys Glu Gly Met Ser Arg His Ala Thr Phe Glu Asn Phe Gly Met Ala
1620 1625 1630

Phe Leu Thr Leu Phe Gln Val Ser Thr Gly Asp Asn Trp Asn Gly Ile
1635 1640 1645

Met Lys Asp Thr Leu Arg Asp Cys Thr His Asp Glu Arg Ser Cys Leu
1650 1655 1660

Ser Ser Leu Gln Phe Val Ser Pro Leu Tyr Phe Val Ser Phe Val Leu
1665 1670 1675 1680

Thr Ala Gln Phe Val Leu Ile Asn Val Val Val Ala Val Leu Met Lys
1685 1690 1695

His Leu Asp Asp Ser Asn Lys Glu Ala Gln Glu Asp Ala Glu Met Asp
1700 1705 1710

Ala Glu Leu Glu Leu Glu Met Ala His Gly Leu Gly Pro Gly Pro Arg
1715 1720 1725

Leu Pro Thr Gly Ser Pro Gly Ala Pro Gly Arg Gly Pro Gly Gly Ala
1730 1735 1740

Gly Gly Gly Gly Asp Thr Glu Gly Gly Leu Cys Arg Arg Cys Tyr Ser
1745 1750 1755 1760

Pro Ala Gln Asp Ser Leu Glu Gly Glu Leu Thr Ile Ile Asp Asn Leu
1765 1770 1775

Ser Gly Ser Ile Phe His His Tyr Ser Ser Pro Ala Gly Cys Lys Lys
1780 1785 1790

Cys His His Asp Lys Gln Glu Val Gln Leu Ala Glu Thr Glu Ala Phe
1795 1800 1805

Ser Leu Asn Ser Asp Arg Ser Ser Ser Ile Leu Leu Gly Asp Asp Leu

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    1810                1815                1820

Ser Leu Glu Asp Pro Thr Ala Cys Pro Pro Gly Arg Lys Asp Ser Lys
1825                1830                1835                1840

Gly Glu Leu Asp Pro Pro Glu Pro Met Arg Val Gly Asp Leu Gly Glu
                1845                1850                1855

Cys Phe Phe Pro Leu Ser Ser Thr Ala Val Ser Pro Asp Pro Glu Asn
            1860                1865                1870

Phe Leu Cys Glu Met Glu Glu Ile Pro Phe Asn Pro Val Arg Ser Trp
        1875                1880                1885

Leu Lys His Asp Ser Ser Gln Ala Pro Pro Ser Pro Phe Ser Pro Asp
    1890                1895                1900

Ala Ser Ser Pro Leu Leu Pro Met Pro Ala Glu Phe Phe His Pro Ala
1905                1910                1915                1920

Val Ser Ala Ser Gln Lys Gly Pro Glu Lys Gly Thr Gly Thr Gly Thr
                1925                1930                1935

Leu Pro Lys Ile Ala Leu Gln Gly Ser Trp Ala Ser Leu Arg Ser Pro
            1940                1945                1950

Arg Val Asn Cys Thr Leu Leu Arg Gln Ala Thr Gly Ser Asp Thr Ser
        1955                1960                1965

Leu Asp Ala Ser Pro Ser Ser Ser Ala Gly Ser Leu Gln Thr Thr Leu
    1970                1975                1980

Glu Asp Ser Leu Thr Leu Ser Asp Ser Pro Arg Arg Ala Leu Gly Pro
1985                1990                1995                2000

Pro Ala Pro Ala Pro Gly Pro Arg Ala Gly Leu Ser Pro Ala Ala Arg
                2005                2010                2015

Arg Arg Leu Ser Leu Arg Gly Arg Gly Leu Phe Ser Leu Arg Gly Leu
            2020                2025                2030

Arg Ala His Gln Arg Ser His Ser Ser Gly Gly Ser Thr Ser Pro Gly
        2035                2040                2045

Cys Thr His His Asp Ser Met Asp Pro Ser Asp Glu Glu Gly Arg Gly
    2050                2055                2060

Gly Ala Gly Gly Gly Gly Ala Gly Ser Glu His Ser Glu Thr Leu Ser
2065                2070                2075                2080

Ser Leu Ser Leu Thr Ser Leu Phe Cys Pro Pro Pro Pro Pro Pro Ala
                2085                2090                2095

Pro Gly Leu Thr Pro Ala Arg Lys Phe Ser Ser Thr Ser Ser Leu Ala
            2100                2105                2110

Ala Pro Gly Arg Pro His Ala Ala Ala Leu Ala His Gly Leu Ala Arg
        2115                2120                2125

Ser Pro Ser Trp Ala Ala Asp Arg Ser Lys Asp Pro Pro Gly Arg Ala
    2130                2135                2140

Pro Leu Pro Met Gly Leu Gly Pro Leu Ala Pro Pro Pro Gln Pro Leu
2145                2150                2155                2160

Pro Gly Glu Leu Glu Pro Gly Asp Ala Ala Ser Lys Arg Lys Arg
                2165                2170                2175


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<211> LENGTH: 6855
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
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<400> SEQUENCE: 3
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tcaggtgggg ctgtcagagc tgcatccgtc cacttattgg tggagaggca ggttggggag     120

catgtaccag gcctgtcccc accacgtgcc accctctctg tcttccccag ggctcccagc     180

tcagtgtgga c atg gct gag agc gcc tcc ccg ccc tcc tca tct gca gca       230
            Met Ala Glu Ser Ala Ser Pro Pro Ser Ser Ser Ala Ala
              1               5                  10

gcc cca gcc gct gag cca gga gtc acc acg gag cag ccc gga ccc cgg       278
Ala Pro Ala Ala Glu Pro Gly Val Thr Thr Glu Gln Pro Gly Pro Arg
     15                  20                  25

agc ccc cca tcc tcc ccg cca ggc ctg gag gag cct ctg gat gga gct       326
Ser Pro Pro Ser Ser Pro Pro Gly Leu Glu Glu Pro Leu Asp Gly Ala
 30                  35                  40                  45

gat cct cat gtc cca cac cca gac ctg gcg cct att gcc ttc ttc tgc       374
Asp Pro His Val Pro His Pro Asp Leu Ala Pro Ile Ala Phe Phe Cys
                 50                  55                  60

ctg cga cag acc acc agc ccc cgg aac tgg tgc atc aag atg gtg tgc       422
Leu Arg Gln Thr Thr Ser Pro Arg Asn Trp Cys Ile Lys Met Val Cys
            65                  70                  75

aac ccg tgg ttt gaa tgt gtc agc atg ctg gtg atc ctg ctg aac tgc       470
Asn Pro Trp Phe Glu Cys Val Ser Met Leu Val Ile Leu Leu Asn Cys
         80                  85                  90

gtg aca ctt ggc atg tac cag ccg tgc gac gac atg gac tgc ctg tcc       518
Val Thr Leu Gly Met Tyr Gln Pro Cys Asp Asp Met Asp Cys Leu Ser
     95                  100                 105

gac cgc tgc aag atc ctg cag gtc ttt gat gac ttc atc ttt atc ttc       566
Asp Arg Cys Lys Ile Leu Gln Val Phe Asp Asp Phe Ile Phe Ile Phe
110                 115                 120                 125

ttt gcc atg gag atg gtg ctc aag atg gtg gcc ctg ggg att ttt ggc       614
Phe Ala Met Glu Met Val Leu Lys Met Val Ala Leu Gly Ile Phe Gly
                130                 135                 140

aag aag tgc tac ctc ggg gac aca tgg aac cgc ctg gat ttc ttc atc       662
Lys Lys Cys Tyr Leu Gly Asp Thr Trp Asn Arg Leu Asp Phe Phe Ile
            145                 150                 155

gtc atg gca ggg atg gtc gag tac tcc ctg gac ctt cag aac atc aac       710
Val Met Ala Gly Met Val Glu Tyr Ser Leu Asp Leu Gln Asn Ile Asn
        160                 165                 170

ctg tca gcc atc cgc acc gtg cgc gtc ctg agg ccc ctc aaa gcc atc       758
Leu Ser Ala Ile Arg Thr Val Arg Val Leu Arg Pro Leu Lys Ala Ile
    175                 180                 185

aac cgc gtg ccc agt atg cgg atc ctg gtg aac ctg ctc ctg gac aca       806
Asn Arg Val Pro Ser Met Arg Ile Leu Val Asn Leu Leu Leu Asp Thr
190                 195                 200                 205

ctg ccc atg ctg ggg aat gtc ctg ctg ctc tgc ttc ttt gtc ttc ttc       854
Leu Pro Met Leu Gly Asn Val Leu Leu Leu Cys Phe Phe Val Phe Phe
                210                 215                 220

atc ttt ggc atc ata ggt gtg cag ctc tgg gcg ggc ctg ctg cgt aac       902
Ile Phe Gly Ile Ile Gly Val Gln Leu Trp Ala Gly Leu Leu Arg Asn
            225                 230                 235

cgc tgc ttc ctg gag gag aac ttc acc ata caa ggg gat gtg gcc ttg       950
Arg Cys Phe Leu Glu Glu Asn Phe Thr Ile Gln Gly Asp Val Ala Leu
        240                 245                 250

ccc cca tac tac cag ccg gag gag gat gat gag atg ccc ttc atc tgc       998
Pro Pro Tyr Tyr Gln Pro Glu Glu Asp Asp Glu Met Pro Phe Ile Cys
    255                 260                 265

tcc ctg tcg ggc gac aat ggg ata atg ggc tgc cat gag atc ccc ccg      1046
Ser Leu Ser Gly Asp Asn Gly Ile Met Gly Cys His Glu Ile Pro Pro
270                 275                 280                 285

ctc aag gag cag ggc cgt gag tgc tgc ctg tcc aag gac gac gtc tac      1094
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Leu Lys Glu Gln Gly Arg Glu Cys Cys Leu Ser Lys Asp Asp Val Tyr
                290                 295                 300

gac ttt ggg gcg ggg cgc cag gac ctc aat gcc agc ggc ctc tgt gtc    1142
Asp Phe Gly Ala Gly Arg Gln Asp Leu Asn Ala Ser Gly Leu Cys Val
            305                 310                 315

aac tgg aac cgt tac tac aat gtg tgc cgc acg ggc agc gcc aac ccc    1190
Asn Trp Asn Arg Tyr Tyr Asn Val Cys Arg Thr Gly Ser Ala Asn Pro
        320                 325                 330

cac aag ggt gcc atc aac ttt gac aac atc ggt tat gct tgg att gtc    1238
His Lys Gly Ala Ile Asn Phe Asp Asn Ile Gly Tyr Ala Trp Ile Val
    335                 340                 345

atc ttc cag gtg atc act ctg gaa ggc tgg gtg gag atc atg tac tac    1286
Ile Phe Gln Val Ile Thr Leu Glu Gly Trp Val Glu Ile Met Tyr Tyr
350                 355                 360                 365

gtg atg gat gct cac tcc ttc tac aac ttc atc tac ttc atc ctg ctt    1334
Val Met Asp Ala His Ser Phe Tyr Asn Phe Ile Tyr Phe Ile Leu Leu
                370                 375                 380

atc ata gtg ggc tcc ttc ttc atg atc aac ctg tgc ctc gtt gtc ata    1382
Ile Ile Val Gly Ser Phe Phe Met Ile Asn Leu Cys Leu Val Val Ile
            385                 390                 395

gcg acc cag ttc tcg gag acc aag caa cgg gag cac cgg ctg atg ctg    1430
Ala Thr Gln Phe Ser Glu Thr Lys Gln Arg Glu His Arg Leu Met Leu
        400                 405                 410

gag cag cgg cag cgc tac ctg tcc tcc agc acg gtg gcc agc tac gcc    1478
Glu Gln Arg Gln Arg Tyr Leu Ser Ser Ser Thr Val Ala Ser Tyr Ala
    415                 420                 425

gag cct ggc gac tgc tac gag gag atc ttc cag tat gtc tgc cac atc    1526
Glu Pro Gly Asp Cys Tyr Glu Glu Ile Phe Gln Tyr Val Cys His Ile
430                 435                 440                 445

ctg cgc aag gcc aag cgc cgc gcc ctg ggc ctc tac cag gcc ctg cag    1574
Leu Arg Lys Ala Lys Arg Arg Ala Leu Gly Leu Tyr Gln Ala Leu Gln
                450                 455                 460

agc cgg cgc cag gcc ctg ggc ccg gag gcc ccg gcc ccc gcc aaa cct    1622
Ser Arg Arg Gln Ala Leu Gly Pro Glu Ala Pro Ala Pro Ala Lys Pro
            465                 470                 475

ggg ccc cac gcc aag gag ccc cgg cac tac cag ctg tgc ccg caa cat    1670
Gly Pro His Ala Lys Glu Pro Arg His Tyr Gln Leu Cys Pro Gln His
        480                 485                 490

agc ccc ctg gat gcg acg ccc cac acc ctg gtg cag ccc atc ccc gcc    1718
Ser Pro Leu Asp Ala Thr Pro His Thr Leu Val Gln Pro Ile Pro Ala
    495                 500                 505

acg ctg gct tcc gat ccc gcc agc tgc cct tgc tgc cag cat gag gac    1766
Thr Leu Ala Ser Asp Pro Ala Ser Cys Pro Cys Cys Gln His Glu Asp
510                 515                 520                 525

ggc cgg cgg ccc tcg ggc ctg ggc agc acc gac tcg ggc cag gag ggc    1814
Gly Arg Arg Pro Ser Gly Leu Gly Ser Thr Asp Ser Gly Gln Glu Gly
                530                 535                 540

tcg ggc tcc ggg agc tcc gct ggt ggc gag gac gag gcg gat ggg gac    1862
Ser Gly Ser Gly Ser Ser Ala Gly Gly Glu Asp Glu Ala Asp Gly Asp
            545                 550                 555

ggg gcc cgg agc agc gag gac gga gcc tcc tca gaa ctg ggg aag gag    1910
Gly Ala Arg Ser Ser Glu Asp Gly Ala Ser Ser Glu Leu Gly Lys Glu
        560                 565                 570

gag gag gag gag gag cag gcg gat ggg gcg gtc tgg ctg tgc ggg gat    1958
Glu Glu Glu Glu Glu Gln Ala Asp Gly Ala Val Trp Leu Cys Gly Asp
    575                 580                 585

gtg tgg cgg gag acg cga gcc aag ctg cgc ggc atc gtg gac agc aag    2006
Val Trp Arg Glu Thr Arg Ala Lys Leu Arg Gly Ile Val Asp Ser Lys
590                 595                 600                 605
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tac ttc aac cgg ggc atc atg atg gcc atc ctg gtc aac acc gtc agc     2054
Tyr Phe Asn Arg Gly Ile Met Met Ala Ile Leu Val Asn Thr Val Ser
                610                 615                 620

atg ggc atc gag cac cac gag cag ccg gag gag ctg acc aac atc ctg     2102
Met Gly Ile Glu His His Glu Gln Pro Glu Glu Leu Thr Asn Ile Leu
            625                 630                 635

gag atc tgc aat gtg gtc ttc acc agc atg ttt gcc ctg gag atg atc     2150
Glu Ile Cys Asn Val Val Phe Thr Ser Met Phe Ala Leu Glu Met Ile
        640                 645                 650

ctg aag ctg gct gca ttt ggg ctc ttc gac tac ctg cgt aac ccc tac     2198
Leu Lys Leu Ala Ala Phe Gly Leu Phe Asp Tyr Leu Arg Asn Pro Tyr
    655                 660                 665

aac atc ttc gac agc atc att gtc atc atc agc atc tgg gag atc gtg     2246
Asn Ile Phe Asp Ser Ile Ile Val Ile Ile Ser Ile Trp Glu Ile Val
670                 675                 680                 685

ggg cag gcg gac ggt ggg ctg tcg gtg ctg cgg acc ttc cgg ctg ctg     2294
Gly Gln Ala Asp Gly Gly Leu Ser Val Leu Arg Thr Phe Arg Leu Leu
                690                 695                 700

cgc gtg ctg aaa ctg gtg cgc ttc atg cct gcc ctg cgg cgc cag ctc     2342
Arg Val Leu Lys Leu Val Arg Phe Met Pro Ala Leu Arg Arg Gln Leu
            705                 710                 715

gtg gtg ctc atg aag acc atg gac aac gtg gcc acc ttc tgc atg ctg     2390
Val Val Leu Met Lys Thr Met Asp Asn Val Ala Thr Phe Cys Met Leu
        720                 725                 730

ctc atg ctc ttc atc ttc atc ttc agc atc ctt ggg atg cat att ttt     2438
Leu Met Leu Phe Ile Phe Ile Phe Ser Ile Leu Gly Met His Ile Phe
    735                 740                 745

ggc tgc aag ttc agc ctc cgc acg gac act gga gac acg gtg ccc gac     2486
Gly Cys Lys Phe Ser Leu Arg Thr Asp Thr Gly Asp Thr Val Pro Asp
750                 755                 760                 765

agg aag aac ttc gac tcc ctg ctg tgg gcc atc gtc act gtg ttc cag     2534
Arg Lys Asn Phe Asp Ser Leu Leu Trp Ala Ile Val Thr Val Phe Gln
                770                 775                 780

atc ctc acc cag gag gac tgg aac gtc gtt ctc tac aat ggc atg gcc     2582
Ile Leu Thr Gln Glu Asp Trp Asn Val Val Leu Tyr Asn Gly Met Ala
            785                 790                 795

tcc act tct ccc tgg gcc tcc ctc tac ttt gtc gcc ctc atg acc ttc     2630
Ser Thr Ser Pro Trp Ala Ser Leu Tyr Phe Val Ala Leu Met Thr Phe
        800                 805                 810

ggc aac tat gtg ctc ttc aac ctg ctg gtg gcc atc ctg gtg gag ggc     2678
Gly Asn Tyr Val Leu Phe Asn Leu Leu Val Ala Ile Leu Val Glu Gly
    815                 820                 825

ttc cag gcg gag ggt gac gcc aat cgc tcc tac tcg gac gag gac cag     2726
Phe Gln Ala Glu Gly Asp Ala Asn Arg Ser Tyr Ser Asp Glu Asp Gln
830                 835                 840                 845

agc tca tcc aac ata gaa gag ttt gat aag ctc cag gaa ggc ctg gac     2774
Ser Ser Ser Asn Ile Glu Glu Phe Asp Lys Leu Gln Glu Gly Leu Asp
                850                 855                 860

agc agc gga gat ccc aag ctc tgc cca atc ccc atg acc ccc aat ggg     2822
Ser Ser Gly Asp Pro Lys Leu Cys Pro Ile Pro Met Thr Pro Asn Gly
            865                 870                 875

cac ctg gac ccc agt ctc cca ctg ggt ggg cac cta ggt cct gct ggg     2870
His Leu Asp Pro Ser Leu Pro Leu Gly Gly His Leu Gly Pro Ala Gly
        880                 885                 890

gct gcg gga cct gcc ccc cga ctc tca ctg cag ccg gac ccc atg ctg     2918
Ala Ala Gly Pro Ala Pro Arg Leu Ser Leu Gln Pro Asp Pro Met Leu
    895                 900                 905

gtg gcc ctg ggc tcc cga aag agc agt gtc atg tct cta ggg agg atg     2966
Val Ala Leu Gly Ser Arg Lys Ser Ser Val Met Ser Leu Gly Arg Met
910                 915                 920                 925
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agc tat gac cag cgc tcc ctg tcc agc tcc cgg agc tcc tac tac ggg     3014
Ser Tyr Asp Gln Arg Ser Leu Ser Ser Ser Arg Ser Ser Tyr Tyr Gly
                930                 935                 940

cca tgg ggc cgc agc gcg gcc tgg gcc agc cgt cgc tcc agc tgg aac     3062
Pro Trp Gly Arg Ser Ala Ala Trp Ala Ser Arg Arg Ser Ser Trp Asn
            945                 950                 955

agc ctc aag cac aag ccg ccg tcg gcg gag cat gag tcc ctg ctc tct     3110
Ser Leu Lys His Lys Pro Pro Ser Ala Glu His Glu Ser Leu Leu Ser
        960                 965                 970

gcg gag cgc ggc ggc ggc gcc cgg gtc tgc gag gtt gcc gcg gac gag     3158
Ala Glu Arg Gly Gly Gly Ala Arg Val Cys Glu Val Ala Ala Asp Glu
    975                 980                 985

ggg ccg ccg cgg gcc gca ccc ctg cac acc cca cac gcc cac cac att     3206
Gly Pro Pro Arg Ala Ala Pro Leu His Thr Pro His Ala His His Ile
990                 995                 1000                1005

cat cac ggg ccc cat ctg gcg cac cgc cac cgc cac cac cgc cgg acg     3254
His His Gly Pro His Leu Ala His Arg His Arg His His Arg Arg Thr
                1010                1015                1020

ctg tcc ctc gac aac agg gac tcg gtg gac ctg gcc gag ctg gtg ccc     3302
Leu Ser Leu Asp Asn Arg Asp Ser Val Asp Leu Ala Glu Leu Val Pro
            1025                1030                1035

gcg gtg ggc gcc cac ccc cgg gcc gcc tgg agg gcg gca ggc ccg gcc     3350
Ala Val Gly Ala His Pro Arg Ala Ala Trp Arg Ala Ala Gly Pro Ala
        1040                1045                1050

ccc ggg cat gag gac tgc aat ggc agg atg ccc agc atc gcc aaa gac     3398
Pro Gly His Glu Asp Cys Asn Gly Arg Met Pro Ser Ile Ala Lys Asp
    1055                1060                1065

gtc ttc acc aag atg ggc gac cgc ggg gat cgc ggg gag gat gag gag     3446
Val Phe Thr Lys Met Gly Asp Arg Gly Asp Arg Gly Glu Asp Glu Glu
1070                1075                1080                1085

gaa atc gac tac acc ctg tgc ttc cgc gtc cgc aag atg atc gac gtc     3494
Glu Ile Asp Tyr Thr Leu Cys Phe Arg Val Arg Lys Met Ile Asp Val
                1090                1095                1100

tat aag ccc gac tgg tgc gag gtc cgc gaa gac tgg tct gtc tac ctc     3542
Tyr Lys Pro Asp Trp Cys Glu Val Arg Glu Asp Trp Ser Val Tyr Leu
            1105                1110                1115

ttc tct ccc gag aac agg ttc cgg gtc ctg tgt cag acc att att gcc     3590
Phe Ser Pro Glu Asn Arg Phe Arg Val Leu Cys Gln Thr Ile Ile Ala
        1120                1125                1130

cac aaa ctc ttc gac tac gtc gtc ctg gcc ttc atc ttt ctc aac tgc     3638
His Lys Leu Phe Asp Tyr Val Val Leu Ala Phe Ile Phe Leu Asn Cys
    1135                1140                1145

atc acc atc gcc ctg gag cgg cct cag atc gag gcc ggc agc acc gaa     3686
Ile Thr Ile Ala Leu Glu Arg Pro Gln Ile Glu Ala Gly Ser Thr Glu
1150                1155                1160                1165

cgc atc ttt ctc acc gtg tcc aac tac atc ttc acg gcc atc ttc gtg     3734
Arg Ile Phe Leu Thr Val Ser Asn Tyr Ile Phe Thr Ala Ile Phe Val
                1170                1175                1180

ggc gag atg aca ttg aag gta gtc tcg ctg ggc ctg tac ttc ggc gag     3782
Gly Glu Met Thr Leu Lys Val Val Ser Leu Gly Leu Tyr Phe Gly Glu
            1185                1190                1195

cag gcg tac cta cgc agc agc tgg aac gtg ctg gat ggc ttt ctt gtc     3830
Gln Ala Tyr Leu Arg Ser Ser Trp Asn Val Leu Asp Gly Phe Leu Val
        1200                1205                1210

ttc gtg tcc atc atc gac atc gtg gtg tcc ctg gcc tca gcc ggg gga     3878
Phe Val Ser Ile Ile Asp Ile Val Val Ser Leu Ala Ser Ala Gly Gly
    1215                1220                1225

gcc aag atc ttg ggg gtc ctc cga gtc ttg cgg ctc ctg cgc acc cta     3926
Ala Lys Ile Leu Gly Val Leu Arg Val Leu Arg Leu Leu Arg Thr Leu
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1230                1235                1240                1245

cgc ccc ctg cgt gtc atc agc cgg gcg ccg ggc ctg aag ctg gtg gtg     3974
Arg Pro Leu Arg Val Ile Ser Arg Ala Pro Gly Leu Lys Leu Val Val
                1250                1255                1260

gag aca ctc atc tcc tcc ctc aag ccc atc ggc aac atc gtg ctc atc     4022
Glu Thr Leu Ile Ser Ser Leu Lys Pro Ile Gly Asn Ile Val Leu Ile
            1265                1270                1275

tgc tgt gcc ttc ttc atc atc ttt ggc atc ctg gga gtg cag ctc ttc     4070
Cys Cys Ala Phe Phe Ile Ile Phe Gly Ile Leu Gly Val Gln Leu Phe
        1280                1285                1290

aag ggc aag ttc tac cac tgt ctg ggc gtg gac acc cgc aac atc acc     4118
Lys Gly Lys Phe Tyr His Cys Leu Gly Val Asp Thr Arg Asn Ile Thr
    1295                1300                1305

aac cgc tcg gac tgc atg gcc gcc aac tac cgc tgg gtc cat cac aaa     4166
Asn Arg Ser Asp Cys Met Ala Ala Asn Tyr Arg Trp Val His His Lys
1310                1315                1320                1325

tac aac ttc gac aac ctg ggc cag gct ctg atg tcc ctc ttt gtc ctg     4214
Tyr Asn Phe Asp Asn Leu Gly Gln Ala Leu Met Ser Leu Phe Val Leu
                1330                1335                1340

gca tcc aag gat ggt tgg gtg aac atc atg tac aat gga ctg gat gct     4262
Ala Ser Lys Asp Gly Trp Val Asn Ile Met Tyr Asn Gly Leu Asp Ala
            1345                1350                1355

gtt gct gtg gac cag cag cct gtg acc aac cac aac ccc tgg atg ctg     4310
Val Ala Val Asp Gln Gln Pro Val Thr Asn His Asn Pro Trp Met Leu
        1360                1365                1370

ctg tac ttc atc tcc ttc ctg ctc atc gtc agc ttc ttt gtg ctc aac     4358
Leu Tyr Phe Ile Ser Phe Leu Leu Ile Val Ser Phe Phe Val Leu Asn
    1375                1380                1385

atg ttt gtg ggt gtc gtg gtg gag aac ttc cac aag tgc cgg cag cac     4406
Met Phe Val Gly Val Val Val Glu Asn Phe His Lys Cys Arg Gln His
1390                1395                1400                1405

cag gag gct gaa gag gca cgg cgg cgt gag gag aag cgg ctg cgg cgc     4454
Gln Glu Ala Glu Glu Ala Arg Arg Arg Glu Glu Lys Arg Leu Arg Arg
                1410                1415                1420

ctg gag aag aag cgc cgg aag gcc cag cgg ctg ccc tac tat gcc acc     4502
Leu Glu Lys Lys Arg Arg Lys Ala Gln Arg Leu Pro Tyr Tyr Ala Thr
            1425                1430                1435

tat tgt cac acc cgg ctg ctc atc cac tcc atg tgc acc agc cac tac     4550
Tyr Cys His Thr Arg Leu Leu Ile His Ser Met Cys Thr Ser His Tyr
        1440                1445                1450

ctg gac atc ttc atc acc ttc atc atc tgc ctc aac gtg gtc acc atg     4598
Leu Asp Ile Phe Ile Thr Phe Ile Ile Cys Leu Asn Val Val Thr Met
    1455                1460                1465

tcc ctg gag cac tac aat cag ccc acg tcc ctg gag aca gcc ctc aag     4646
Ser Leu Glu His Tyr Asn Gln Pro Thr Ser Leu Glu Thr Ala Leu Lys
1470                1475                1480                1485

tac tgc aac tat atg ttc acc act gtc ttt gtg ctg gag gct gtg ctg     4694
Tyr Cys Asn Tyr Met Phe Thr Thr Val Phe Val Leu Glu Ala Val Leu
                1490                1495                1500

aag ctg gtg gca ttt ggt ctg agg cgc ttc ttc aag gac cga tgg aac     4742
Lys Leu Val Ala Phe Gly Leu Arg Arg Phe Phe Lys Asp Arg Trp Asn
            1505                1510                1515

cag ctg gac ctg gcc att gtg cta ctg tca gtc atg ggc atc acc ctg     4790
Gln Leu Asp Leu Ala Ile Val Leu Leu Ser Val Met Gly Ile Thr Leu
        1520                1525                1530

gag gag atc gag atc aat gcg gcc ctg ccc atc aat ccc acc atc atc     4838
Glu Glu Ile Glu Ile Asn Ala Ala Leu Pro Ile Asn Pro Thr Ile Ile
    1535                1540                1545

cgc atc atg agg gtt ctg cgc att gcc cga gtg ctg aag ctg ttg aag     4886
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Arg Ile Met Arg Val Leu Arg Ile Ala Arg Val Leu Lys Leu Leu Lys
1550                1555                1560                1565

atg gcc aca gga atg cgg gcc ctg ctg gac acg gtg gtg caa gct ttg     4934
Met Ala Thr Gly Met Arg Ala Leu Leu Asp Thr Val Val Gln Ala Leu
                1570                1575                1580

ccc cag gtg ggc aac ctg ggc ctc ctc ttc atg ctg ctc ttc ttc atc     4982
Pro Gln Val Gly Asn Leu Gly Leu Leu Phe Met Leu Leu Phe Phe Ile
            1585                1590                1595

tat gct gct ctc ggg gtg gag ctc ttt ggg aag ctg gtc tgc aac gac     5030
Tyr Ala Ala Leu Gly Val Glu Leu Phe Gly Lys Leu Val Cys Asn Asp
        1600                1605                1610

gag aac ccg tgc gag ggc atg agc cgg cat gcc acc ttc gag aac ttc     5078
Glu Asn Pro Cys Glu Gly Met Ser Arg His Ala Thr Phe Glu Asn Phe
    1615                1620                1625

ggc atg gcc ttc ctc aca ctc ttc cag gtc tcc acg ggt gac aac tgg     5126
Gly Met Ala Phe Leu Thr Leu Phe Gln Val Ser Thr Gly Asp Asn Trp
1630                1635                1640                1645

aac ggg atc atg aag gac acg ctg cgg gac tgc acc cac gac gag cgc     5174
Asn Gly Ile Met Lys Asp Thr Leu Arg Asp Cys Thr His Asp Glu Arg
                1650                1655                1660

agc tgc ctg agc agc ctg cag ttt gtg tcg ccg ctg tac ttc gtg agc     5222
Ser Cys Leu Ser Ser Leu Gln Phe Val Ser Pro Leu Tyr Phe Val Ser
            1665                1670                1675

ttc gtg ctc acc gcg cag ttc gtg ctc atc aac gtg gtg gtg gct gtg     5270
Phe Val Leu Thr Ala Gln Phe Val Leu Ile Asn Val Val Val Ala Val
        1680                1685                1690

ctc atg aag cac ctg gac gac agc aac aag gag gcg cag gag gac gcc     5318
Leu Met Lys His Leu Asp Asp Ser Asn Lys Glu Ala Gln Glu Asp Ala
    1695                1700                1705

gag atg gat gcc gag ctc gag ctg gag atg gcc cat ggc ctg ggc cct     5366
Glu Met Asp Ala Glu Leu Glu Leu Glu Met Ala His Gly Leu Gly Pro
1710                1715                1720                1725

ggc ccg agg ctg cct acc ggc tcc ccg ggc gcc cct ggc cga ggg ccg     5414
Gly Pro Arg Leu Pro Thr Gly Ser Pro Gly Ala Pro Gly Arg Gly Pro
                1730                1735                1740

gga ggg gcg ggc ggc ggg ggc gac acc gag ggc ggc ttg tgc cgg cgc     5462
Gly Gly Ala Gly Gly Gly Gly Asp Thr Glu Gly Gly Leu Cys Arg Arg
            1745                1750                1755

tgc tac tcg cct gcc cag gag aac ctg tgg ctg gac agc gtc tct tta     5510
Cys Tyr Ser Pro Ala Gln Glu Asn Leu Trp Leu Asp Ser Val Ser Leu
        1760                1765                1770

atc atc aag gac tcc ttg gag ggg gag ctg acc atc atc gac aac ctg     5558
Ile Ile Lys Asp Ser Leu Glu Gly Glu Leu Thr Ile Ile Asp Asn Leu
    1775                1780                1785

tcg ggc tcc atc ttc cac cac tac tcc tcg cct gcc ggc tgc aag aag     5606
Ser Gly Ser Ile Phe His His Tyr Ser Ser Pro Ala Gly Cys Lys Lys
1790                1795                1800                1805

tgt cac cac gac aag caa gag gtg cag ctg gct gag acg gag gcc ttc     5654
Cys His His Asp Lys Gln Glu Val Gln Leu Ala Glu Thr Glu Ala Phe
                1810                1815                1820

tcc ctg aac tca gac agg tcc tcg tcc atc ctg ctg ggt gac gac ctg     5702
Ser Leu Asn Ser Asp Arg Ser Ser Ser Ile Leu Leu Gly Asp Asp Leu
            1825                1830                1835

agt ctc gag gac ccc aca gcc tgc cca cct ggc cgc aaa gac agc aag     5750
Ser Leu Glu Asp Pro Thr Ala Cys Pro Pro Gly Arg Lys Asp Ser Lys
        1840                1845                1850

ggt gag ctg gac cca cct gag ccc atg cgt gtg gga gac ctg ggc gaa     5798
Gly Glu Leu Asp Pro Pro Glu Pro Met Arg Val Gly Asp Leu Gly Glu
    1855                1860                1865
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tgc ttc ttc ccc ttg tcc tct acg gcc gtc tcg ccg gat cca gag aac     5846
Cys Phe Phe Pro Leu Ser Ser Thr Ala Val Ser Pro Asp Pro Glu Asn
1870                1875                1880                1885

ttc ctg tgt gag atg gag gag atc cca ttc aac cct gtc cgg tcc tgg     5894
Phe Leu Cys Glu Met Glu Glu Ile Pro Phe Asn Pro Val Arg Ser Trp
                1890                1895                1900

ctg aaa cat gac agc agt caa gca ccc cca agt ccc ttc tcc ccg gat     5942
Leu Lys His Asp Ser Ser Gln Ala Pro Pro Ser Pro Phe Ser Pro Asp
            1905                1910                1915

gcc tcc agc cct ctc ctg ccc atg cca gcc gag ttc ttc cac cct gca     5990
Ala Ser Ser Pro Leu Leu Pro Met Pro Ala Glu Phe Phe His Pro Ala
        1920                1925                1930

gtg tct gcc agc cag aaa ggc cca gaa aag ggc act ggc act gga acc     6038
Val Ser Ala Ser Gln Lys Gly Pro Glu Lys Gly Thr Gly Thr Gly Thr
    1935                1940                1945

ctc ccc aag att gcg ctg cag ggc tcc tgg gca tct ctg cgg tca cca     6086
Leu Pro Lys Ile Ala Leu Gln Gly Ser Trp Ala Ser Leu Arg Ser Pro
1950                1955                1960                1965

agg gtc aac tgt acc ctc ctc cgg cag gcc acc ggg agc gac acg tcg     6134
Arg Val Asn Cys Thr Leu Leu Arg Gln Ala Thr Gly Ser Asp Thr Ser
                1970                1975                1980

ctg gac gcc agc ccc agc agc tcc gcg ggc agc ctg cag acc acg ctc     6182
Leu Asp Ala Ser Pro Ser Ser Ser Ala Gly Ser Leu Gln Thr Thr Leu
            1985                1990                1995

gag gac agc ctg acc ctg agc gac agc ccc cgg cgt gcc ctg ggg ccg     6230
Glu Asp Ser Leu Thr Leu Ser Asp Ser Pro Arg Arg Ala Leu Gly Pro
        2000                2005                2010

ccc gcg cct gct cca gga ccc cgg gcc ggc ctg tcc ccc gcc gct cgc     6278
Pro Ala Pro Ala Pro Gly Pro Arg Ala Gly Leu Ser Pro Ala Ala Arg
    2015                2020                2025

cgc cgc ctg agc ctg cgc ggc cgg ggc ctc ttc agc ctg cgg ggg ctg     6326
Arg Arg Leu Ser Leu Arg Gly Arg Gly Leu Phe Ser Leu Arg Gly Leu
2030                2035                2040                2045

cgg gcg cat cag cgc agc cac agc agc ggg ggc tcc acc agc ccg ggc     6374
Arg Ala His Gln Arg Ser His Ser Ser Gly Gly Ser Thr Ser Pro Gly
                2050                2055                2060

tgc acc cac cac gac tcc atg gac ccc tcg gac gag gag ggc cgc ggt     6422
Cys Thr His His Asp Ser Met Asp Pro Ser Asp Glu Glu Gly Arg Gly
            2065                2070                2075

ggc gcg ggc ggc ggg ggc gcg ggc agc gag cac tcg gag acc ctc agc     6470
Gly Ala Gly Gly Gly Gly Ala Gly Ser Glu His Ser Glu Thr Leu Ser
        2080                2085                2090

agc ctc tcg ctc acc tcc ctc ttc tgc ccg ccg ccc ccg ccg cca gcc     6518
Ser Leu Ser Leu Thr Ser Leu Phe Cys Pro Pro Pro Pro Pro Pro Ala
    2095                2100                2105

ccc ggc ctc acg ccc gcc agg aag ttc agc agc acc agc agc ctg gcc     6566
Pro Gly Leu Thr Pro Ala Arg Lys Phe Ser Ser Thr Ser Ser Leu Ala
2110                2115                2120                2125

gcc ccc ggc cgc ccc cac gcc gcc gcc ctg gcc cac ggc ctg gcc cgg     6614
Ala Pro Gly Arg Pro His Ala Ala Ala Leu Ala His Gly Leu Ala Arg
                2130                2135                2140

agc ccc tcg tgg gcc gcg gac cgc agc aag gac ccc ccc ggc cgg gca     6662
Ser Pro Ser Trp Ala Ala Asp Arg Ser Lys Asp Pro Pro Gly Arg Ala
            2145                2150                2155

ccg ctg ccc atg ggc ctg ggc ccc ttg gcg ccc ccg ccg caa ccg ctc     6710
Pro Leu Pro Met Gly Leu Gly Pro Leu Ala Pro Pro Pro Gln Pro Leu
        2160                2165                2170

ccc gga gag ctg gag ccg gga gac gcc gcc agc aag agg aag aga         6755
Pro Gly Glu Leu Glu Pro Gly Asp Ala Ala Ser Lys Arg Lys Arg
    2175                2180                2185
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tgagggtcgc aggggcccc ggccgcccac cgcccgcccc gtctcacctt ctttacctca 6815

ggagccagga gcagacagca atacttcgtc cacacctggg 6855

<210> SEQ ID NO 4
<211> LENGTH: 2188
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

Met Ala Glu Ser Ala Ser Pro Pro Ser Ser Ser Ala Ala Ala Pro Ala
1 5 10 15

Ala Glu Pro Gly Val Thr Thr Glu Gln Pro Gly Pro Arg Ser Pro Pro
20 25 30

Ser Ser Pro Pro Gly Leu Glu Glu Pro Leu Asp Gly Ala Asp Pro His
35 40 45

Val Pro His Pro Asp Leu Ala Pro Ile Ala Phe Phe Cys Leu Arg Gln
50 55 60

Thr Thr Ser Pro Arg Asn Trp Cys Ile Lys Met Val Cys Asn Pro Trp
65 70 75 80

Phe Glu Cys Val Ser Met Leu Val Ile Leu Leu Asn Cys Val Thr Leu
85 90 95

Gly Met Tyr Gln Pro Cys Asp Asp Met Asp Cys Leu Ser Asp Arg Cys
100 105 110

Lys Ile Leu Gln Val Phe Asp Asp Phe Ile Phe Ile Phe Phe Ala Met
115 120 125

Glu Met Val Leu Lys Met Val Ala Leu Gly Ile Phe Gly Lys Lys Cys
130 135 140

Tyr Leu Gly Asp Thr Trp Asn Arg Leu Asp Phe Phe Ile Val Met Ala
145 150 155 160

Gly Met Val Glu Tyr Ser Leu Asp Leu Gln Asn Ile Asn Leu Ser Ala
165 170 175

Ile Arg Thr Val Arg Val Leu Arg Pro Leu Lys Ala Ile Asn Arg Val
180 185 190

Pro Ser Met Arg Ile Leu Val Asn Leu Leu Leu Asp Thr Leu Pro Met
195 200 205

Leu Gly Asn Val Leu Leu Leu Cys Phe Phe Val Phe Phe Ile Phe Gly
210 215 220

Ile Ile Gly Val Gln Leu Trp Ala Gly Leu Leu Arg Asn Arg Cys Phe
225 230 235 240

Leu Glu Glu Asn Phe Thr Ile Gln Gly Asp Val Ala Leu Pro Pro Tyr
245 250 255

Tyr Gln Pro Glu Glu Asp Asp Glu Met Pro Phe Ile Cys Ser Leu Ser
260 265 270

Gly Asp Asn Gly Ile Met Gly Cys His Glu Ile Pro Pro Leu Lys Glu
275 280 285

Gln Gly Arg Glu Cys Cys Leu Ser Lys Asp Asp Val Tyr Asp Phe Gly
290 295 300

Ala Gly Arg Gln Asp Leu Asn Ala Ser Gly Leu Cys Val Asn Trp Asn
305 310 315 320

Arg Tyr Tyr Asn Val Cys Arg Thr Gly Ser Ala Asn Pro His Lys Gly
325 330 335

Ala Ile Asn Phe Asp Asn Ile Gly Tyr Ala Trp Ile Val Ile Phe Gln
340 345 350

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Val Ile Thr Leu Glu Gly Trp Val Glu Ile Met Tyr Tyr Val Met Asp
        355                 360                 365

Ala His Ser Phe Tyr Asn Phe Ile Tyr Phe Ile Leu Leu Ile Ile Val
    370                 375                 380

Gly Ser Phe Phe Met Ile Asn Leu Cys Leu Val Val Ile Ala Thr Gln
385                 390                 395                 400

Phe Ser Glu Thr Lys Gln Arg Glu His Arg Leu Met Leu Glu Gln Arg
                405                 410                 415

Gln Arg Tyr Leu Ser Ser Ser Thr Val Ala Ser Tyr Ala Glu Pro Gly
            420                 425                 430

Asp Cys Tyr Glu Glu Ile Phe Gln Tyr Val Cys His Ile Leu Arg Lys
        435                 440                 445

Ala Lys Arg Arg Ala Leu Gly Leu Tyr Gln Ala Leu Gln Ser Arg Arg
    450                 455                 460

Gln Ala Leu Gly Pro Glu Ala Pro Ala Pro Ala Lys Pro Gly Pro His
465                 470                 475                 480

Ala Lys Glu Pro Arg His Tyr Gln Leu Cys Pro Gln His Ser Pro Leu
                485                 490                 495

Asp Ala Thr Pro His Thr Leu Val Gln Pro Ile Pro Ala Thr Leu Ala
            500                 505                 510

Ser Asp Pro Ala Ser Cys Pro Cys Cys Gln His Glu Asp Gly Arg Arg
        515                 520                 525

Pro Ser Gly Leu Gly Ser Thr Asp Ser Gly Gln Glu Gly Ser Gly Ser
    530                 535                 540

Gly Ser Ser Ala Gly Gly Glu Asp Glu Ala Asp Gly Asp Gly Ala Arg
545                 550                 555                 560

Ser Ser Glu Asp Gly Ala Ser Ser Glu Leu Gly Lys Glu Glu Glu Glu
                565                 570                 575

Glu Glu Gln Ala Asp Gly Ala Val Trp Leu Cys Gly Asp Val Trp Arg
            580                 585                 590

Glu Thr Arg Ala Lys Leu Arg Gly Ile Val Asp Ser Lys Tyr Phe Asn
        595                 600                 605

Arg Gly Ile Met Met Ala Ile Leu Val Asn Thr Val Ser Met Gly Ile
    610                 615                 620

Glu His His Glu Gln Pro Glu Glu Leu Thr Asn Ile Leu Glu Ile Cys
625                 630                 635                 640

Asn Val Val Phe Thr Ser Met Phe Ala Leu Glu Met Ile Leu Lys Leu
                645                 650                 655

Ala Ala Phe Gly Leu Phe Asp Tyr Leu Arg Asn Pro Tyr Asn Ile Phe
            660                 665                 670

Asp Ser Ile Ile Val Ile Ile Ser Ile Trp Glu Ile Val Gly Gln Ala
        675                 680                 685

Asp Gly Gly Leu Ser Val Leu Arg Thr Phe Arg Leu Leu Arg Val Leu
    690                 695                 700

Lys Leu Val Arg Phe Met Pro Ala Leu Arg Arg Gln Leu Val Val Leu
705                 710                 715                 720

Met Lys Thr Met Asp Asn Val Ala Thr Phe Cys Met Leu Leu Met Leu
                725                 730                 735

Phe Ile Phe Ile Phe Ser Ile Leu Gly Met His Ile Phe Gly Cys Lys
            740                 745                 750

Phe Ser Leu Arg Thr Asp Thr Gly Asp Thr Val Pro Asp Arg Lys Asn
        755                 760                 765

Phe Asp Ser Leu Leu Trp Ala Ile Val Thr Val Phe Gln Ile Leu Thr
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    770                 775                 780

Gln Glu Asp Trp Asn Val Val Leu Tyr Asn Gly Met Ala Ser Thr Ser
785                 790                 795                 800

Pro Trp Ala Ser Leu Tyr Phe Val Ala Leu Met Thr Phe Gly Asn Tyr
                805                 810                 815

Val Leu Phe Asn Leu Leu Val Ala Ile Leu Val Glu Gly Phe Gln Ala
            820                 825                 830

Glu Gly Asp Ala Asn Arg Ser Tyr Ser Asp Glu Asp Gln Ser Ser Ser
        835                 840                 845

Asn Ile Glu Glu Phe Asp Lys Leu Gln Glu Gly Leu Asp Ser Ser Gly
    850                 855                 860

Asp Pro Lys Leu Cys Pro Ile Pro Met Thr Pro Asn Gly His Leu Asp
865                 870                 875                 880

Pro Ser Leu Pro Leu Gly Gly His Leu Gly Pro Ala Gly Ala Ala Gly
                885                 890                 895

Pro Ala Pro Arg Leu Ser Leu Gln Pro Asp Pro Met Leu Val Ala Leu
            900                 905                 910

Gly Ser Arg Lys Ser Ser Val Met Ser Leu Gly Arg Met Ser Tyr Asp
        915                 920                 925

Gln Arg Ser Leu Ser Ser Ser Arg Ser Ser Tyr Tyr Gly Pro Trp Gly
    930                 935                 940

Arg Ser Ala Ala Trp Ala Ser Arg Arg Ser Ser Trp Asn Ser Leu Lys
945                 950                 955                 960

His Lys Pro Pro Ser Ala Glu His Glu Ser Leu Leu Ser Ala Glu Arg
                965                 970                 975

Gly Gly Gly Ala Arg Val Cys Glu Val Ala Ala Asp Glu Gly Pro Pro
            980                 985                 990

Arg Ala Ala Pro Leu His Thr Pro His Ala His His Ile His His Gly
        995                 1000                1005

Pro His Leu Ala His Arg His Arg His His Arg Arg Thr Leu Ser Leu
    1010                1015                1020

Asp Asn Arg Asp Ser Val Asp Leu Ala Glu Leu Val Pro Ala Val Gly
1025                1030                1035                1040

Ala His Pro Arg Ala Ala Trp Arg Ala Ala Gly Pro Ala Pro Gly His
                1045                1050                1055

Glu Asp Cys Asn Gly Arg Met Pro Ser Ile Ala Lys Asp Val Phe Thr
            1060                1065                1070

Lys Met Gly Asp Arg Gly Asp Arg Gly Glu Asp Glu Glu Glu Ile Asp
        1075                1080                1085

Tyr Thr Leu Cys Phe Arg Val Arg Lys Met Ile Asp Val Tyr Lys Pro
    1090                1095                1100

Asp Trp Cys Glu Val Arg Glu Asp Trp Ser Val Tyr Leu Phe Ser Pro
1105                1110                1115                1120

Glu Asn Arg Phe Arg Val Leu Cys Gln Thr Ile Ile Ala His Lys Leu
                1125                1130                1135

Phe Asp Tyr Val Val Leu Ala Phe Ile Phe Leu Asn Cys Ile Thr Ile
            1140                1145                1150

Ala Leu Glu Arg Pro Gln Ile Glu Ala Gly Ser Thr Glu Arg Ile Phe
        1155                1160                1165

Leu Thr Val Ser Asn Tyr Ile Phe Thr Ala Ile Phe Val Gly Glu Met
    1170                1175                1180

Thr Leu Lys Val Val Ser Leu Gly Leu Tyr Phe Gly Glu Gln Ala Tyr
1185                1190                1195                1200
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Leu Arg Ser Ser Trp Asn Val Leu Asp Gly Phe Leu Val Phe Val Ser
1205 1210 1215

Ile Ile Asp Ile Val Val Ser Leu Ala Ser Ala Gly Gly Ala Lys Ile
1220 1225 1230

Leu Gly Val Leu Arg Val Leu Arg Leu Leu Arg Thr Leu Arg Pro Leu
1235 1240 1245

Arg Val Ile Ser Arg Ala Pro Gly Leu Lys Leu Val Val Glu Thr Leu
1250 1255 1260

Ile Ser Ser Leu Lys Pro Ile Gly Asn Ile Val Leu Ile Cys Cys Ala
1265 1270 1275 1280

Phe Phe Ile Ile Phe Gly Ile Leu Gly Val Gln Leu Phe Lys Gly Lys
1285 1290 1295

Phe Tyr His Cys Leu Gly Val Asp Thr Arg Asn Ile Thr Asn Arg Ser
1300 1305 1310

Asp Cys Met Ala Ala Asn Tyr Arg Trp Val His His Lys Tyr Asn Phe
1315 1320 1325

Asp Asn Leu Gly Gln Ala Leu Met Ser Leu Phe Val Leu Ala Ser Lys
1330 1335 1340

Asp Gly Trp Val Asn Ile Met Tyr Asn Gly Leu Asp Ala Val Ala Val
1345 1350 1355 1360

Asp Gln Gln Pro Val Thr Asn His Asn Pro Trp Met Leu Leu Tyr Phe
1365 1370 1375

Ile Ser Phe Leu Leu Ile Val Ser Phe Phe Val Leu Asn Met Phe Val
1380 1385 1390

Gly Val Val Val Glu Asn Phe His Lys Cys Arg Gln His Gln Glu Ala
1395 1400 1405

Glu Glu Ala Arg Arg Arg Glu Glu Lys Arg Leu Arg Arg Leu Glu Lys
1410 1415 1420

Lys Arg Arg Lys Ala Gln Arg Leu Pro Tyr Tyr Ala Thr Tyr Cys His
1425 1430 1435 1440

Thr Arg Leu Leu Ile His Ser Met Cys Thr Ser His Tyr Leu Asp Ile
1445 1450 1455

Phe Ile Thr Phe Ile Ile Cys Leu Asn Val Val Thr Met Ser Leu Glu
1460 1465 1470

His Tyr Asn Gln Pro Thr Ser Leu Glu Thr Ala Leu Lys Tyr Cys Asn
1475 1480 1485

Tyr Met Phe Thr Thr Val Phe Val Leu Glu Ala Val Leu Lys Leu Val
1490 1495 1500

Ala Phe Gly Leu Arg Arg Phe Phe Lys Asp Arg Trp Asn Gln Leu Asp
1505 1510 1515 1520

Leu Ala Ile Val Leu Leu Ser Val Met Gly Ile Thr Leu Glu Glu Ile
1525 1530 1535

Glu Ile Asn Ala Ala Leu Pro Ile Asn Pro Thr Ile Ile Arg Ile Met
1540 1545 1550

Arg Val Leu Arg Ile Ala Arg Val Leu Lys Leu Leu Lys Met Ala Thr
1555 1560 1565

Gly Met Arg Ala Leu Leu Asp Thr Val Val Gln Ala Leu Pro Gln Val
1570 1575 1580

Gly Asn Leu Gly Leu Leu Phe Met Leu Leu Phe Phe Ile Tyr Ala Ala
1585 1590 1595 1600

Leu Gly Val Glu Leu Phe Gly Lys Leu Val Cys Asn Asp Glu Asn Pro
1605 1610 1615

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Cys Glu Gly Met Ser Arg His Ala Thr Phe Glu Asn Phe Gly Met Ala
1620 1625 1630

Phe Leu Thr Leu Phe Gln Val Ser Thr Gly Asp Asn Trp Asn Gly Ile
1635 1640 1645

Met Lys Asp Thr Leu Arg Asp Cys Thr His Asp Glu Arg Ser Cys Leu
1650 1655 1660

Ser Ser Leu Gln Phe Val Ser Pro Leu Tyr Phe Val Ser Phe Val Leu
1665 1670 1675 1680

Thr Ala Gln Phe Val Leu Ile Asn Val Val Val Ala Val Leu Met Lys
1685 1690 1695

His Leu Asp Asp Ser Asn Lys Glu Ala Gln Glu Asp Ala Glu Met Asp
1700 1705 1710

Ala Glu Leu Glu Leu Glu Met Ala His Gly Leu Gly Pro Gly Pro Arg
1715 1720 1725

Leu Pro Thr Gly Ser Pro Gly Ala Pro Gly Arg Gly Pro Gly Gly Ala
1730 1735 1740

Gly Gly Gly Gly Asp Thr Glu Gly Gly Leu Cys Arg Arg Cys Tyr Ser
1745 1750 1755 1760

Pro Ala Gln Glu Asn Leu Trp Leu Asp Ser Val Ser Leu Ile Ile Lys
1765 1770 1775

Asp Ser Leu Glu Gly Glu Leu Thr Ile Ile Asp Asn Leu Ser Gly Ser
1780 1785 1790

Ile Phe His His Tyr Ser Ser Pro Ala Gly Cys Lys Lys Cys His His
1795 1800 1805

Asp Lys Gln Glu Val Gln Leu Ala Glu Thr Glu Ala Phe Ser Leu Asn
1810 1815 1820

Ser Asp Arg Ser Ser Ser Ile Leu Leu Gly Asp Asp Leu Ser Leu Glu
1825 1830 1835 1840

Asp Pro Thr Ala Cys Pro Pro Gly Arg Lys Asp Ser Lys Gly Glu Leu
1845 1850 1855

Asp Pro Pro Glu Pro Met Arg Val Gly Asp Leu Gly Glu Cys Phe Phe
1860 1865 1870

Pro Leu Ser Ser Thr Ala Val Ser Pro Asp Pro Glu Asn Phe Leu Cys
1875 1880 1885

Glu Met Glu Glu Ile Pro Phe Asn Pro Val Arg Ser Trp Leu Lys His
1890 1895 1900

Asp Ser Ser Gln Ala Pro Pro Ser Pro Phe Ser Pro Asp Ala Ser Ser
1905 1910 1915 1920

Pro Leu Leu Pro Met Pro Ala Glu Phe Phe His Pro Ala Val Ser Ala
1925 1930 1935

Ser Gln Lys Gly Pro Glu Lys Gly Thr Gly Thr Gly Thr Leu Pro Lys
1940 1945 1950

Ile Ala Leu Gln Gly Ser Trp Ala Ser Leu Arg Ser Pro Arg Val Asn
1955 1960 1965

Cys Thr Leu Leu Arg Gln Ala Thr Gly Ser Asp Thr Ser Leu Asp Ala
1970 1975 1980

Ser Pro Ser Ser Ser Ala Gly Ser Leu Gln Thr Thr Leu Glu Asp Ser
1985 1990 1995 2000

Leu Thr Leu Ser Asp Ser Pro Arg Arg Ala Leu Gly Pro Pro Ala Pro
2005 2010 2015

Ala Pro Gly Pro Arg Ala Gly Leu Ser Pro Ala Ala Arg Arg Arg Leu
2020 2025 2030

Ser Leu Arg Gly Arg Gly Leu Phe Ser Leu Arg Gly Leu Arg Ala His

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        2035                2040                2045

Gln Arg Ser His Ser Ser Gly Gly Ser Thr Ser Pro Gly Cys Thr His
    2050                2055                2060

His Asp Ser Met Asp Pro Ser Asp Glu Glu Gly Arg Gly Gly Ala Gly
2065                2070                2075                2080

Gly Gly Gly Ala Gly Ser Glu His Ser Glu Thr Leu Ser Ser Leu Ser
                2085                2090                2095

Leu Thr Ser Leu Phe Cys Pro Pro Pro Pro Pro Pro Ala Pro Gly Leu
            2100                2105                2110

Thr Pro Ala Arg Lys Phe Ser Ser Thr Ser Ser Leu Ala Ala Pro Gly
        2115                2120                2125

Arg Pro His Ala Ala Ala Leu Ala His Gly Leu Ala Arg Ser Pro Ser
    2130                2135                2140

Trp Ala Ala Asp Arg Ser Lys Asp Pro Pro Gly Arg Ala Pro Leu Pro
2145                2150                2155                2160

Met Gly Leu Gly Pro Leu Ala Pro Pro Pro Gln Pro Leu Pro Gly Glu
                2165                2170                2175

Leu Glu Pro Gly Asp Ala Ala Ser Lys Arg Lys Arg
            2180                2185


<210> SEQ ID NO 5
<211> LENGTH: 1835
<212> TYPE: PRT
<213> ORGANISM: Rattus sp.

<400> SEQUENCE: 5

Met Ala Asp Ser Asn Leu Pro Pro Ser Ser Ala Ala Ala Pro Ala Pro
  1               5                  10                  15

Glu Pro Gly Ile Thr Glu Gln Pro Gly Pro Arg Ser Pro Pro Pro Ser
            20                  25                  30

Pro Pro Gly Leu Glu Glu Pro Leu Glu Gly Thr Asn Pro Asp Val Pro
        35                  40                  45

His Pro Asp Leu Ala Pro Val Ala Phe Phe Cys Leu Arg Gln Thr Thr
    50                  55                  60

Ser Pro Arg Asn Trp Cys Ile Lys Met Val Cys Asn Pro Trp Phe Glu
65                  70                  75                  80

Cys Val Ser Met Leu Val Ile Leu Leu Asn Cys Val Thr Leu Gly Met
                85                  90                  95

Tyr Gln Pro Cys Asp Asp Met Glu Cys Leu Ser Asp Arg Cys Lys Ile
            100                 105                 110

Leu Gln Val Phe Asp Asp Phe Ile Phe Ile Phe Phe Ala Met Glu Met
        115                 120                 125

Val Leu Lys Met Val Ala Leu Gly Ile Phe Gly Lys Lys Cys Tyr Leu
    130                 135                 140

Gly Asp Thr Trp Asn Arg Leu Asp Phe Phe Ile Val Met Ala Gly Met
145                 150                 155                 160

Val Glu Tyr Ser Leu Asp Leu Gln Asn Ile Asn Leu Ser Ala Ile Arg
                165                 170                 175

Thr Val Arg Val Leu Arg Pro Leu Lys Ala Ile Asn Arg Val Pro Ser
            180                 185                 190

Met Arg Ile Leu Val Asn Leu Leu Leu Asp Thr Leu Pro Met Leu Gly
        195                 200                 205

Asn Val Leu Leu Leu Cys Phe Phe Val Phe Phe Ile Phe Gly Ile Ile
    210                 215                 220
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Gly Val Gln Leu Trp Ala Gly Leu Leu Arg Asn Arg Cys Phe Leu Glu
225                 230                 235                 240

Glu Asn Phe Thr Ile Gln Gly Asp Val Ala Leu Pro Pro Tyr Tyr Gln
                245                 250                 255

Pro Glu Glu Asp Asp Glu Met Pro Phe Ile Cys Ser Leu Thr Gly Asp
            260                 265                 270

Asn Gly Ile Met Gly Cys His Glu Ile Pro Pro Leu Lys Glu Gln Gly
        275                 280                 285

Arg Glu Cys Cys Leu Ser Lys Asp Asp Val Tyr Asp Phe Gly Ala Gly
    290                 295                 300

Arg Gln Asp Leu Asn Ala Ser Gly Leu Cys Val Asn Trp Asn Arg Tyr
305                 310                 315                 320

Tyr Asn Val Cys Arg Thr Gly Asn Ala Asn Pro His Lys Gly Ala Ile
                325                 330                 335

Asn Phe Asp Asn Ile Gly Tyr Ala Gly Ile Val Ile Phe Gln Val Ile
            340                 345                 350

Thr Leu Glu Gly Trp Val Glu Ile Met Tyr Tyr Val Met Asp Ala His
        355                 360                 365

Ser Phe Tyr Asn Phe Ile Tyr Phe Ile Leu Leu Ile Ile Val Gly Ser
    370                 375                 380

Phe Phe Met Ile Asn Leu Cys Leu Val Val Ile Ala Thr Gln Phe Ser
385                 390                 395                 400

Glu Thr Lys Gln Arg Glu His Arg Leu Met Leu Glu Gln Arg Gln Arg
                405                 410                 415

Tyr Leu Ser Ser Ser Thr Val Ala Ser Tyr Ala Glu Pro Gly Asp Cys
            420                 425                 430

Tyr Glu Glu Ile Phe Gln Tyr Val Cys His Ile Leu Arg Lys Ala Lys
        435                 440                 445

Arg Arg Ala Leu Gly Leu Tyr Gln Ala Leu Gln Asn Arg Arg Gln Ala
    450                 455                 460

Met Gly Pro Gly Thr Pro Ala Pro Ala Lys Pro Gly Pro His Ala Lys
465                 470                 475                 480

Glu Pro Ser His Cys Lys Leu Cys Pro Arg His Ser Pro Leu Asp Pro
                485                 490                 495

Thr Pro His Thr Leu Val Gln Pro Ile Ser Ala Ile Leu Ala Ser Asp
            500                 505                 510

Pro Ser Ser Cys Pro His Cys Gln His Glu Ala Gly Arg Arg Pro Ser
        515                 520                 525

Gly Leu Gly Ser Thr Asp Ser Gly Gln Glu Gly Ser Gly Ser Gly Gly
    530                 535                 540

Ser Ala Glu Ala Glu Ala Asn Gly Asp Gly Leu Gln Ser Ser Glu Asp
545                 550                 555                 560

Gly Val Ser Ser Asp Leu Gly Lys Glu Glu Glu Gln Glu Asp Gly Ala
                565                 570                 575

Ala Arg Leu Cys Gly Asp Val Trp Arg Glu Thr Arg Lys Lys Leu Arg
            580                 585                 590

Gly Ile Val Asp Ser Lys Tyr Phe Asn Arg Gly Ile Met Met Ala Ile
        595                 600                 605

Leu Val Asn Thr Val Ser Met Gly Ile Glu His His Glu Gln Pro Glu
    610                 615                 620

Glu Leu Thr Asn Ile Leu Glu Ile Cys Asn Val Val Phe Thr Ser Met
625                 630                 635                 640

Phe Ala Leu Glu Met Ile Leu Lys Leu Ala Ala Phe Gly Leu Phe Asp
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645 650 655

Tyr Leu Arg Asn Pro Tyr Asn Ile Phe Asp Ser Ile Ile Val Ile Ile
660 665 670

Ser Ile Trp Glu Ile Val Gly Gln Ala Asp Gly Gly Leu Ser Val Leu
675 680 685

Arg Thr Phe Arg Leu Leu Arg Val Leu Lys Leu Val Arg Phe Met Pro
690 695 700

Ala Leu Arg Arg Gln Leu Val Val Leu Met Lys Thr Met Asp Asn Val
705 710 715 720

Ala Thr Phe Cys Met Leu Leu Met Leu Phe Ile Phe Ile Phe Ser Ile
725 730 735

Leu Gly Met His Ile Phe Gly Cys Lys Phe Ser Leu Arg Thr Asp Thr
740 745 750

Gly Asp Thr Val Pro Asp Arg Lys Asn Phe Asp Ser Leu Leu Trp Ala
755 760 765

Ile Val Thr Val Phe Gln Ile Leu Thr Gln Glu Asp Trp Asn Val Val
770 775 780

Leu Tyr Asn Gly Met Ala Ser Thr Thr Pro Trp Ala Ser Leu Tyr Phe
785 790 795 800

Val Ala Leu Met Thr Phe Gly Asn Tyr Val Leu Phe Asn Leu Leu Val
805 810 815

Ala Ile Leu Val Glu Gly Phe Gln Ala Glu Gly Asp Ala Asn Arg Ser
820 825 830

Cys Ser Asp Glu Asp Gln Ser Ser Ser Asn Leu Glu Glu Phe Asp Lys
835 840 845

Leu Pro Glu Gly Leu Asp Asn Ser Arg Asp Leu Lys Leu Cys Pro Ile
850 855 860

Pro Met Thr Pro Asn Gly His Leu Asp Pro Ser Leu Pro Leu Gly Ala
865 870 875 880

His Leu Gly Pro Ala Gly Thr Met Gly Thr Ala Pro Arg Leu Ser Leu
885 890 895

Gln Pro Asp Pro Val Leu Val Ala Leu Asp Ser Arg Lys Ser Ser Val
900 905 910

Met Ser Leu Gly Arg Met Ser Tyr Asp Gln Arg Ser Leu Ser Ser Ser
915 920 925

Arg Ser Ser Tyr Tyr Gly Pro Trp Gly Arg Ser Gly Thr Trp Ala Ser
930 935 940

Arg Arg Ser Ser Trp Asn Ser Leu Lys His Lys Pro Pro Ser Ala Glu
945 950 955 960

His Glu Ser Leu Leu Ser Gly Glu Gly Gly Gly Ser Cys Val Arg Ala
965 970 975

Cys Glu Gly Ala Arg Glu Glu Ala Pro Thr Arg Thr Ala Pro Leu His
980 985 990

Ala Pro His Ala His His Ala His His Gly Pro His Leu Ala His Arg
995 1000 1005

His Arg His His Arg Arg Thr Leu Ser Leu Asp Thr Arg Asp Ser Val
1010 1015 1020

Asp Leu Gly Glu Leu Val Pro Val Val Gly Ala His Ser Arg Ala Ala
1025 1030 1035 1040

Trp Arg Gly Ala Gly Gln Ala Pro Gly His Glu Asp Cys Asn Gly Arg
1045 1050 1055

Met Pro Asn Ile Ala Lys Asp Val Phe Thr Lys Met Asp Asp Arg Arg
1060 1065 1070

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Asp Arg Gly Glu Asp Glu Glu Glu Ile Asp Tyr Thr Leu Cys Phe Arg
1075 1080 1085

Val Arg Lys Met Ile Asp Val Tyr Lys Pro Asp Trp Cys Glu Val Arg
1090 1095 1100

Glu Asp Trp Ser Val Tyr Leu Phe Ser Pro Glu Asn Lys Phe Arg Ile
1105 1110 1115 1120

Leu Cys Gln Thr Ile Ile Ala His Lys Leu Phe Asp Tyr Val Val Leu
1125 1130 1135

Ala Phe Ile Phe Leu Asn Cys Ile Thr Ile Ala Leu Glu Arg Pro Gln
1140 1145 1150

Ile Glu Ala Gly Ser Thr Glu Arg Ile Phe Leu Thr Val Ser Asn Tyr
1155 1160 1165

Ile Phe Thr Ala Ile Phe Val Gly Glu Met Thr Leu Lys Val Val Ser
1170 1175 1180

Leu Gly Leu Tyr Phe Gly Glu Gln Ala Tyr Leu Arg Ser Ser Trp Asn
1185 1190 1195 1200

Val Leu Asp Gly Phe Leu Val Phe Val Ser Ile Ile Asp Ile Val Val
1205 1210 1215

Ser Val Ala Ser Ala Gly Gly Ala Lys Ile Leu Gly Val Leu Arg Val
1220 1225 1230

Leu Arg Leu Leu Arg Thr Leu Arg Pro Leu Arg Val Ile Ser Arg Ala
1235 1240 1245

Pro Gly Leu Lys Leu Val Val Glu Thr Leu Ile Ser Ser Leu Lys Pro
1250 1255 1260

Ile Gly Asn Ile Val Leu Ile Cys Cys Ala Phe Phe Ile Ile Phe Gly
1265 1270 1275 1280

Ile Leu Gly Val Gln Leu Phe Lys Gly Lys Phe Tyr His Cys Leu Gly
1285 1290 1295

Val Asp Thr Arg Asn Ile Thr Asn Arg Ser Asp Cys Val Ala Ala Asn
1300 1305 1310

Tyr Arg Trp Val His His Lys Tyr Asn Phe Asp Asn Leu Gly Gln Ala
1315 1320 1325

Leu Met Ser Leu Phe Val Leu Ala Ser Lys Asp Gly Trp Val Asn Ile
1330 1335 1340

Met Tyr Asn Gly Leu Asp Ala Val Ala Val Asp Gln Gln Pro Val Thr
1345 1350 1355 1360

Asn His Asn Pro Trp Met Leu Leu Tyr Phe Ile Ser Phe Leu Leu Ile
1365 1370 1375

Val Ser Phe Phe Val Leu Asn Met Phe Val Gly Val Val Val Glu Asn
1380 1385 1390

Phe His Lys Cys Arg Gln His Gln Glu Ala Glu Glu Ala Arg Arg Arg
1395 1400 1405

Glu Glu Lys Arg Leu Arg Arg Leu Glu Lys Lys Arg Arg Lys Ala Gln
1410 1415 1420

Arg Leu Pro Tyr Tyr Ala Thr Tyr Cys Pro Thr Arg Leu Leu Ile His
1425 1430 1435 1440

Ser Met Cys Thr Ser His Tyr Leu Asp Ile Phe Ile Thr Phe Ile Ile
1445 1450 1455

Cys Leu Asn Val Val Thr Met Ser Leu Glu His Tyr Asn Gln Pro Thr
1460 1465 1470

Ser Leu Glu Thr Ala Leu Lys Tyr Cys Asn Tyr Met Phe Thr Thr Val
1475 1480 1485

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Phe Val Leu Glu Ala Val Leu Lys Leu Val Ala Phe Gly Leu Arg Arg
1490 1495 1500

Phe Phe Lys Asp Arg Trp Asn Gln Leu Asp Leu Ala Ile Val Leu Leu
1505 1510 1515 1520

Ser Val Met Gly Ile Thr Leu Glu Glu Ile Glu Ile Asn Ala Ala Leu
1525 1530 1535

Pro Ile Asn Pro Thr Ile Ile Arg Ile Met Arg Val Leu Arg Ile Ala
1540 1545 1550

Arg Val Leu Lys Leu Leu Lys Met Ala Thr Gly Met Arg Ala Leu Leu
1555 1560 1565

Asp Thr Val Val Gln Ala Leu Pro Gln Val Gly Asn Leu Gly Leu Leu
1570 1575 1580

Phe Met Leu Leu Phe Phe Ile Tyr Ala Ala Leu Gly Val Glu Leu Phe
1585 1590 1595 1600

Gly Lys Leu Val Cys Asn Asp Glu Asn Pro Cys Glu Gly Met Ser Arg
1605 1610 1615

His Ala Thr Phe Glu Asn Phe Gly Met Ala Phe Leu Thr Leu Phe Gln
1620 1625 1630

Val Ser Thr Gly Asp Asn Trp Asn Gly Ile Met Lys Asp Thr Leu Arg
1635 1640 1645

Asp Cys Thr His Asp Glu Arg Thr Cys Leu Ser Ser Leu Gln Phe Val
1650 1655 1660

Ser Pro Leu Tyr Phe Val Ser Phe Val Leu Thr Ala Gln Phe Val Leu
1665 1670 1675 1680

Ile Asn Val Val Val Ala Val Leu Met Lys His Leu Asp Asp Ser Asn
1685 1690 1695

Lys Glu Ala Gln Glu Asp Ala Glu Met Asp Ala Glu Ile Glu Leu Glu
1700 1705 1710

Met Ala His Gly Leu Gly Pro Cys Pro Gly Pro Cys Pro Gly Pro Cys
1715 1720 1725

Pro Cys Pro Cys Pro Cys Pro Cys Ala Gly Pro Arg Leu Pro Thr Ser
1730 1735 1740

Ser Pro Gly Ala Pro Gly Arg Gly Ser Gly Gly Ala Gly Ala Gly Gly
1745 1750 1755 1760

Asp Thr Glu Ser His Leu Cys Arg His Cys Tyr Ser Pro Ala Gln Glu
1765 1770 1775

Thr Leu Trp Leu Asp Ser Val Ser Leu Ile Ile Lys Asp Ser Leu Glu
1780 1785 1790

Gly Glu Leu Thr Ile Ile Asp Asn Leu Ser Gly Ser Val Phe His His
1795 1800 1805

Tyr Ala Ser Pro Asp Gly Cys Gly Lys Cys His His Asp Lys Gln Glu
1810 1815 1820

Thr Gly Leu His Pro Ser Cys Trp Gly Met Thr
1825 1830 1835

<210> SEQ ID NO 6
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6

ctcacgaagt acagcggcga cac 23

<210> SEQ ID NO 7
<211> LENGTH: 25

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<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 7

gggcgccatc aactttgaca acatc 25

<210> SEQ ID NO 8
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 8

ctgggccctc agctgtttcg taatc 25

<210> SEQ ID NO 9
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 9

gcgctggtca tagctcatcc tccctagaga 30

<210> SEQ ID NO 10
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 10

gcgcttcttc aaggaccgat gg 22

<210> SEQ ID NO 11
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 11

cccaggtgtg gacgaagtat tgct 24

<210> SEQ ID NO 12
<211> LENGTH: 6503
<212> TYPE: DNA
<213> ORGANISM: Rattus sp.

<400> SEQUENCE: 12

cggtccgccg ctcgtccgcg ccacccgcct cgagccgcgc ggacccgccc gccatggccc 60

gcgccccccgg gcccgccgcc ctgcatgcgc cgtccccctc gccccggggg cgcagctgat 120

cccggaatcc gaggcgtggg gccggcgggg cgcggggtcc ctctccacgc cggcttcggg 180

gacacgcgtc aaccccgcgt ctctgcccgg gacgaccccg ctgcccggcc acgtccatgc 240

caagggctcc ctgctccacg ctgacatggc tgacagcaac ttaccgccct catctgcagc 300

agccccggcc cctgagccgg gaatcactga gcagccgggg ccccggagtc cccctccatc 360

ccctccaggc ctggaggagc cattggaagg aaccaaccct gacgtcccac atccagacct 420

ggctcctgtt gctttcttct gcctgcgcca gaccacgagc ccacggaact ggtgcatcaa 480

gatggtttgt aacccgtggt tcgagtgtgt gagcatgctg gttattctgc tgaactgtgt 540

gaccctgggc atgtaccagc catgtgatga catggagtgc ctgtcggacc gttgcaagat 600

cctgcaggtc ttcgatgact tcatcttcat cttctttgcc atggagatgg tgcttaagat 660

ggtggccctg ggcatttttg gcaagaagtg ctacctcgga gacacatgga accgcctgga 720

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cccctgcccc tgtgctggcc cgaggctgcc cactagttca cctggggctc cggggcgagg	5520
atcgggaggg gcaggtgctg gaggcgacac cgagagtcac ctgtgccggc actgctattc	5580
tccagcccag gagaccctgt ggctggacag cgtctcttta atcatcaagg actccttgga	5640
gggggagctg accatcattg acaacctgtc tgggtccgtc ttccaccact acgcctcacc	5700
tgacggctgt ggcaagtgtc accatgacaa gcaagagaca ggtcttcatc catcctgctg	5760
ggggatgacc tgagtcttga ggaccccacg gcctgcccac agggccccaa ggagagcaag	5820
ggtgaactag agcctccgga gcccatgcag gctggagacc tggatgaatg cttttggccc	5880
tttgccaagc gagccagtgt ccacaggccc agagagcctg ctgtgcgaga tggggccat	5940
tccattcaac cctgtccagt cctggctcaa acacgagagc agccaagcac cccagagccc	6000
tttctccccg gatggctcca gccctctcct gtagatgcct gctgagttct tccaccctgc	6060
tgtgtctgcc agccagaagg ggcaggaacc gggcatgagt gcaggaaccc tgcccaagat	6120
tgcacttcag gggtcctggg catcgctgag gtcaccgagt gtcaactgca ccctcttgcg	6180
ccaggctact gtgagtgaca cgtccttgga tgccagtcct agcagctcag cgggcagcct	6240
acagaccaca ctggaagaca gtctgactct gagtgacagt ccccggcgtg ccctggggcc	6300
gccggtccag gtgcctgggc cacgggctag cctgtcaccg gccaccccgg cgccgcctca	6360
gcctgcgggg ccgtggcctg tttagtctgc gtgggctgcg ggcccatcag cgtagccaca	6420
gcagtggcgg ctccaccagc cctggctgca ctcaccacga ctccatggac ccctctgatg	6480
aggagggccg cgggggagca ggt	6503

What is claimed is:

1. A polynucleotide encoding a polypeptide comprising the amino acid sequence of SEQ ID NO:2 or4.

2. The polynucleotide of claim 1, wherein the polynucleotide is detectably labeled.

3. An isolated polynucleotide which is the complement of the polynucleotide of claim 1.

4. The isolated polynucleotide of claim 3, wherein the polynucleotide is detectably labeled.

5. The polynucleotide of claim 1, wherein the polynucleotide comprises the nucleic acid sequence of SEQ ID NOs:1 or 3.

6. An expression vector comprising the polynucleotide of claim 1.

7. A host cell comprising the expression vector of claim 6.

8. The host cell of claim 7, wherein the host cell is a prokaryotic cell.

9. The host cell of claim 7, wherein the host cell is a eukaryotic cell.

10. A method of producing an TCCV-1 or TCCV-2 polypeptide, the method comprising:

a) culturing the host cell of claim 7 under conditions suitable for expression of the polypeptide; and

b) recovering the polypeptide from the host cell.

11. A method of detecting a polynucleotide encoding an TCCV-1 or TCCV-2 polypeptide in a sample containing nucleic acid material, the method comprising:

a) contacting the sample with the polynucleotide of claim 3 under conditions suitable for formation of a hybridization complex; and

b) detecting the complex, wherein the presence of the complex is indicative of the presence of the polynucleotide encoding the polypeptide in the sample.

12. A test kit comprising a polynucleotide of claim 5.

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